Stress responses of Escherichia coli

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Stress responses of *Escherichia coli*

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ABSTRACT

Recently, there has been much concern that exposure to biocides in the domestic environment may lead to multiple antibiotic resistance in bacterial pathogens. In the environment bacteria are commonly in a slow growing state, often as an adherent biofilm. Under these conditions, there is another aspect of resistance that is potentially significant, the *rpoS*-regulated general stress response (GSR). The expression of *rpoS* in response to nutrient limitation of *Escherichia coli* was examined. Under conditions of biofilm growth *rpoS* was expressed in all nutrient limitations tested. However, in planktonic culture *rpoS* was expressed only in response to magnesium and phosphate limitation. The influence of *rpoS*, nutrient limitation and cell density on physiological changes associated with stationary phase and resistance to stress was examined. RpoS regulated positively LuxS-mediated quorum sensing and influenced the production of cyclopropane fatty acids, trehalose and resistance to heat. However, the influence of *rpoS* was overridden by conditions of nitrogen limitation or by growth as a biofilm. High cell density was linked to resistance to the biocide triclosan. Overall the specific nutrient limitation had a greater influence on physiological changes and resistance to stress than either *rpoS* expression or cell density. Specific, biofilm-associated phenotypes are proposed to contribute to biofilm resistance. Finally, the relative contribution of the GSR and *marRAB/acrAB* associated resistance in the domestic environment was assessed. Both *acrAB* and *marRAB* were largely uninducible by chemical agents or nutrient limitation.

Key words: *Escherichia coli*, nutrient limitation, biofilms, *rpoS*, *acrAB*, *marRAB*

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Abbreviations

AHL	acylated homoserine lactone
°C	degrees centigrade
CDM	chemically defined medium
CFA	cyclopropane fatty acid
cfu	colony forming units
cm, mm, μm	centimetre, millimeter, micrometre
d, h, min	days, hours, minutes
DNA	deoxyribonucleic acid
FAME	fatty acid methyl ester
g, mg, μg	gram, milligram, microgram
GSR	general stress response
kDa	kilodaltons
l, ml, μl	litre, millilitre, microlitre
LB	Luria-Bertani medium
LM	L-marine medium
MIC	minimum inhibitory concentration
M, mM, μM	molar, millimolar, micromolar
MOPs	(3-(*N*-morpholino) propane sulphonic acid
OD	optical density
Poly P	inorganic polyphosphate
ppGpp	guanosine 5' -diphosphate- 3' –diphosphate
pppGpp	guanosine 5' -triphosphate- 3' –diphosphate
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
x g	times gravitational force
v / v	volume per unit volume

1 INTRODUCTION

Recently, there has been concern that exposure to biocides may induce antibiotic resistance in bacteria. Seminal work by Levy demonstrated that exposure to the disinfectant pine oil resulted in cells displaying a multiple antibiotic resistant (mar) phenotype which included resistance to tetracycline, chloramphenicol and nalidixic acid (Moken et al., 1997). The multiple antibiotic resistant phenotype was found to result from overexpression of the chromosomally encoded *marRAB* operon and the *acrAB* efflux pump (Moken et al., 1997) (discussed below). More recently, research has focused on the widely used biocide triclosan and its role in inducing antibiotic resistance (McMurry et al., 1998a). These findings generated widespread concern that the use of biocides in the hospital and domestic environment could result in multiple antibiotic resistance (summarised in Russell, 1999; Schweizer, 2001).

However, it is important to note that studies of the mar phenotype have been conducted (almost exclusively) under laboratory conditions using rich undefined medium, conditions that do not reflect the natural environmental of most bacteria. The natural environment of enteric bacteria such as *E. coli* is variable but will often include conditions where nutrients are scarce and exposure to stress is frequent. Under these conditions bacteria normally exist in a slow or non-growing state, commonly as a biofilm. Rapid and prolonged exponential growth is uncommon as bacteria rarely encounter the abundant nutrients and benign environment that growth at 37 °C under laboratory conditions affords. Therefore, it can be considered that the study of bacteria under stress conditions is more physiologically relevant than the study of rapid growth (Kolter, 1999).

When bacteria are exposed to stress, there is another aspect of resistance that is potentially significant. Stress, including starvation, adverse environmental conditions and the action of antimicrobial agents may lead to a *rpoS*-regulated, General Stress Response (GSR) (reviewed in Hengge-Aronis, 2000). GSR involves a stationary phase cascade of gene expression, during which structures are protected and the cells become quiescent, a process that resembles sporulation in its physiological consequences. This response is of special significance as it may result in bacteria that are more resistant to numerous stresses and in some cases, more virulent (Hengge-Aronis, 2000). To date, the relative importance of resistance caused by the mar phenotype and the GSR has not been established. In addition, it is important to determine if expression of the mar phenotype and the GSR are influenced not only by biocides but also by the stresses and growth conditions likely to be encountered in the natural environment.

1.1 RpoS-regulated general stress response

The RpoS sigma factor (σ^S) is the master regulator of many stationary phase controlled genes. σ^S is the product of the *rpoS* gene, part of the *nlpD-rpoS* operon, which has two closely spaced promoter sites upstream of *nlpD* (which encodes a lipoprotein of unclear function) (Lange and Hengge-Aronis, 1994). The *rpoS* gene can also be expressed from a promoter actually within the *nlpD*-coding region, this shorter transcript accumulates under certain stationary-phase conditions (Lange et al., 1995). RpoS encodes a protein of 37.8 kDa in common laboratory *E. coli* strains such as MC4100 and W3110 but the locus is highly variable in both natural isolates and long-term agar slope laboratory cultures. Different sized variants are common (Jishage and Ishihama, 1997).

σ^S acts as a sigma subunit of RNA polymerase and is closely related to the housekeeping (vegetative) sigma factor σ^{70} (RpoD). Although once described as a secondary sigma factor, σ^S has now been elevated in status to that of a "second primary" sigma factor to reflect its importance in stationary phase survival and lack of true promoter specificity (Hengge-Aronis, 2000; Lonetto et al., 1992). Although σ^S-dependant changes in gene expression are seen predominantly in stationary phase, this is not exclusively the case with many genes also being induced during the exponential phase (Hengge-Aronis, 1996; Lonetto et al., 1992). In *E. coli* approximately fifty genes are induced under the control of the alternative sigma factor σ^S (Hengge-Aronis, 1996; Loewen et al., 1998; Hengge-Aronis, 1996). The development of *rpoS*-regulated resistance and the associated morphological and physiological changes occur as a result of a programmed change of gene expression. Although the general stress response can be considered in some ways to be analogous to sporulation in certain Gram positive bacteria it differs significantly as it does not involve an irreversible commitment; exponential growth may resume again as soon as environmental conditions improve.

1.1.1 Phenotypic changes related to the general stress response

Physiological consequences of the GSR include: resistance to multiple stresses and structural changes such as accumulation of storage compounds and alteration of the cell envelope. In some cases bacteria may also become more virulent (reviewed in Loewen et al., 1998; Hengge-Aronis, 2000).

1.1.1.1 RpoS and resistance to stress

Induction of σ^S results in the expression of a variety of genes that confer resistance to stress including that of oxidative stress, desiccation, low pH, heat shock and ethanol.

RpoS-negative *E. coli* are considerably more susceptible to many environmental stresses than their wild type parents. In studies of oxidative, osmotic and heat stress, six to thirty-fold differences in the survival rate between RpoS-negative and positive cells have been reported (Lange and Hengge-Aronis, 1991; Hengge-Aronis et al., 1991; McCann et al., 1991; Cheville et al., 1996).

σ^S controlled genes that contribute to resistance to oxidative stress have been reviewed (Eisenstark et al., 1996). Genes such as *dps* that prevent the formation of potentially DNA-damaging hydroxyl free radicals (Almiron et al., 1992) mediate resistance to oxidative stress. Other genes crucial for resistance to hydrogen peroxide and other oxidative stress agents include *xthA*-encoded endonuclease III, the two catalase genes *katG* and *katE* (Sak et al., 1989) and the *sodC* encoded periplasmic superoxide dismutase (Strohmeier-Gort et al., 1999).

σ^S-regulated events also play an important role in osmoprotection. The non-reducing disaccharide trehalose is a compatible solute that has a protective effect on proteins and biological membranes during cryopreservation and desiccation *in vitro* and is implicated in surviving exposure to environmental stress *in vivo* (De Smet et al., 2000). The synthesis of trehalose is regulated by *rpoS* via the genes *otsA* and *otsB* which encode a trehalose-6-phosphate synthase and a trehalose-6-phosphate phosphatase, respectively (Horlacher et al., 1996). *E. coli* contains little or no

trehalose during exponential growth under 'non- stressed' conditions (De Smet et al., 2000). However, trehalose rapidly accumulates in the periplasm in response to high salt (Garcia De Castro et al., 2000), heat shock and at the onset of stationary phase (Hengge-Aronis et al., 1991). In addition, trehalose has been shown to play a minor role in thermotolerance.

It is thought that σ^S plays an uncharacterised role in heat resistance as *rpoS* null mutants are rapidly killed after exposure to temperatures in excess of 50 °C (Lange and Hengge-Aronis, 1991; Cheville et al., 1996). Recently, σ^S has been shown to be involved in resistance to high hydrostatic pressure in *E. coli* O157:H7 but as with many other *rpoS*-regulated events the mechanism is unknown (Robey et al., 2001).

RpoS also has a role in acid resistance in *E. coli* (reviewed in Foster, 2000). However, σ^S-dependent acid resistance is very difficult to distinguish from non-σ^S related events, as the strategies for dealing with acid stress are highly interconnected. Acid resistance (AR) system 1 is expressed in oxidatively metabolising bacteria and is σ^S / CRP-dependent and repressed by glucose, although the mechanism of action is unknown (Lin et al., 1995). AR system 2 has a strict requirement for extracellular glutamate and it has been suggested that there may be an element of σ^S-dependency in this system (Lin et al., 1996). It has been proposed that glutamate decarboxylase and the GABA antiporter *gadC* work in concert to consume intracellular protons thus helping to return the pH to neutral (Castanie-Cornet et al., 1999). However, this system is complicated: although these genes are induced in stationary phase by σ^S, in the exponential phase it appears that other (as yet, undetermined) sigma factors may

be responsible. Another *rpoS*-regulated mechanism of coping with acidic conditions involves the formation of cyclopropane fatty acids (CFAs) (Chang and Cronan, 1999).

CFA formation is a post-synthetic modification of the lipid bilayer that occurs as *E. coli* enters stationary phase (Wang and Cronan, 1994; Grogan and Cronan, 1997). The reaction is catalysed by a soluble enzyme CFA synthase and is under the control of two promoters. The distal promoter P1 is a σ^{70} promoter that is active throughout the growth cycle whereas the proximal P2 promoter is σ^{S} specific and active only during the transition to stationary phase (Wang and Cronan, 1994). As the formation of CFAs is a highly energetic process (Grogan and Cronan, 1997) and the phenomenon is widespread throughout bacteria it seems likely that there are significant physiological consequences of CFA formation that assist bacterial survival during stationary phase. However, widespread studies have been unable to define clearly the physiological role of CFAs. It has been found that CFAs, unlike unsaturated fatty acids, may protect against certain forms of oxidation and assist survival under hyperbaric conditions (Grogan and Cronan, 1997). The production of isogenic wild type and CFA-defective strains has enabled thorough screening of the effects of various chemical and environmental stresses on survival. To date, differences have been observed only after exposure to 20 % (v/v) ethanol, during freeze-thaw treatments (Grogan and Cronan, 1986) and in response to rapid pH drop (Chang and Cronan, 1999; Brown et al., 1997). The correlation between resistance to acid shock (pH3) and CFA content has been hypothesised to be due to decreased proton permeability or increased active proton efflux (Chang and Cronan, 1999).

Resistance to ethanol is regulated by σ^S via the gene *uspB* (Farewell et al., 1998). It is thought that *uspB* encodes a transmembrane protein that confers resistance to ethanol induced membrane damage. However, it is thought that resistance to ethanol involves significant overlap between σ^S and σ^E (extracytoplasmic heat shock sigma factor) -regulated events (Yuru et al., 2000)

It is apparent that the information available on σ^S -regulated stress-protective genes is incomplete. σ^S is known to confer resistance to a wide variety of stresses. However, comparatively few genes have been identified that are responsible and the molecular mode of action for most genes is unknown. In addition, it is important to note that in other species, various environmental factors may have a profound influence on the extent of the requirement for σ^S. However, this has not been widely studied in *E. coli*. It has been shown in *Y. enterocolitica* that the ability of a *rpoS* mutant to survive a variety of stresses was affected at 37 °C but not at 26 °C (Badger and Miller, 1995). Also, the extent of the requirement for *rpoS* in *Sh. flexneri* for stress resistance was shown to depend on environmental factors such as pH or anaerobiosis (Small et al., 1994).

1.1.1.2 The influence of *rpoS* on general morphology and physiology

σ^S is involved in the dramatic changes in cell morphology that occur as cells enter stationary phase (reviewed in Hengge-Aronis , 2000). The physiological changes that result in cells that are smaller and more spherical are regulated at least partly by σ^S through the genes *bolA* and *ftsQAZ*. The gene *bolA* encodes a morphogen that is responsible for round cell morphology (Aldea et al., 1988). One promoter (bolA1p) is σ^S-regulated (Lange and Hengge-Aronis, 1991). Interestingly, it has been found

that expression of *bolA* may be independent of σ^S if cells are exposed to stress during growth (Santos et al., 1999). Similarly, one of the *ftsQ* promoters is σ^S-dependent. It is thought that expression of the *ftsQAZ* operon may be responsible for reductive cell division leading to smaller and more ovoid morphology (Ballesteros et al., 1998).

Many genes that are induced by σ^S influence cell envelope properties and peptidoglycan structure. The genes *osmB*, *C* and *E* are all σ^S-regulated. The functions of *osmC* and *E* are unknown but *osmB* encodes an outer membrane lipoprotein that is implicated in cell aggregation (Jung et al., 1989). The gene *csg* is dependent on σ^S and is responsible for the formation of thin aggregative fimbriae and distinctive colony morphology (Arnqvist et al., 1994). Additionally, *osmY* is strongly induced in response to σ^S and encodes a periplasmic protein of unknown function (Lange et al., 1993). Changes in peptidoglycan are largely due to a turnover process in which penicillin binding proteins (PBP) play a role. Both PBP3 and PBP6 are regulated by *rpoS*; PBP3 negatively and PBP6 positively. PBP6 may be of particular significance as it is thought to play a role in the stabilisation of stationary phase peptidoglycan, analogous to the sporulation specific PBP5a of *Bacillus subtilis* (Huisman et al., 1996).

More recently, σ^S has been implicated in biofilm physiology. It was found that deletion of *rpoS* caused differences in biofilm cell arrangement and significantly reduced biofilm density compared with the parental wild type strain (Adams and McLean, 1999).

1.1.1.3 RpoS and the redirection of metabolism

Major metabolic changes occur at the onset of stationary phase (reviewed in (Huisman et al., 1996; Hengge-Aronis, 2000)). In response to certain types of environmental stress (particularly nutrient limitation) metabolism may be redirected to enable the utilisation of alternative energy sources and the turnover of surplus cellular components. The production of acetyl-CoA synthase (Acs) is σ^S-regulated and enables the reutilization of acetate that is produced and excreted during rapid growth (Shin et al., 1997). The production of pyruvate oxidase (PoxB) enables the production of acetate and carbon dioxide from pyruvate (Chang et al., 1994). An important acid phosphatase, AppA is also σ^S-dependent (Huisman et al., 1996). σ^S also plays a role in the accumulation of glycogen as a carbon source via the putative priming protein GlyS (Huisman et al., 1996) and the turnover of superfluous protein to produce new protein by CbpA. Trehalose may also be utilised as a carbon source if it is no longer required for osmoprotection by the σ^S-regulated cytoplasmic trehalase *treF* (Horlacher et al., 1996).

1.1.1.4 RpoS and virulence

The role of σ^S in colonisation, invasion and intracellular survival has been a subject of much recent study. However, the exact role of *rpoS* in these processes is still unclear: under some circumstances *rpoS* is beneficial but under other conditions the presence of *rpoS* is distinctly disadvantageous. The effect of σ^S on the invasion of brain microvascular endothelial cells (BMEC) by *E.coli* K1 has been examined. It was found that isolates from cerebrospinal fluid had a non-sense mutation in *rpoS* and that complementation with the K12 *rpoS* gene significantly increased invasion of BMEC for some strains (Wang and Kim, 2000). In a similar manner, *rpoS* mutants were

found to have decreased ability to colonise murine Peyer's patches compared with the wild type strain (Fang et al., 1992). However, the evidence for the role of σ^S in colonisation is disputable as other studies have indicated that although *rpoS* plays a role in the initial stages of systemic infection it is not required for infection leading to gastroenteritis in *S. typhimurium* (Nickerson and Curtiss III , 1997). In addition, *rpoS* was found to be unnecessary or even disadvantageous for colonisation of the mouse large intestine by *E. coli* (Krogfelt et al., 2000).

In terms of intracellular invasion and survival, evidence for the role of *rpoS* is also varied. Studies of the intracellular pathogen *Legionella pneumophila* have found that *rpoS* is essential for survival in the environmental protozoan *Acanthomoeba castellani* (Hales and Shuman, 1999) and co-operates with other regulators to induce stationary phase associated virulence (Bachman and Swanson, 2001). However, other studies have found that *rpoS* is not necessary for the colonization and killing of macrophages (Hengge-Aronis, 2000).

Many studies have focused on the role of *rpoS* in the regulation of virulence factors such as exotoxins and adhesins. Recently, expression of the SEF14 fimbriae of *S. enterica* serovar *Enteriditis* has been examined and regulation of this virulence factor was found to be co-ordinated by σ^S (Edwards et al., 2001). RpoS has also been linked to the expression of plasmid-encoded *spv* genes of *S. typhimurium* (Kowartz et al., 1994). In a similar manner, studies of the plant pathogen *Erwinia caratova* and *Ralstonia solanacearum*, found that σ^S was involved in the regulation of virulence genes and the production of exoenzymes (Hengge-Aronis, 2000). Pathogenic *E. coli* strains also use σ^S to regulate virulence genes. In both EHEC and EPEC strains the

esp genes that encode a type III secretion system and are essential for cellular adhesion are reported to be controlled by *rpoS* (Beltrametti et al., 1999). Paradoxically, type 1 fimbriae, which are important for adhesion, are negatively regulated by σ^S (Dove and Dorman, 1994). This negative regulation of type 1 pili may explain why σ^S-dependent gene expression is not essential for the growth of pyelonephritic *E. coli* isolates in human urine or for the colonization of the murine urinary tract (Culham et al., 2001).

σ^S has been shown to be important in lung infection. *P. aeruginosa rpoS* mRNA has been directly isolated from the sputum of cystic fibrosis patients (Foley et al., 1999). However, an *rpoS* mutant of *Pseudomonas aeruginosa* produces less endotoxin A and alginate but showed increased levels of pyocyanin and pyoverdine and possessed enhanced virulence in the rat chronic lung infection model compared with the wild type strain (Suh et al., 1999).

1.1.2 RpoS and the 'GASP' phenotype

Seminal work by Kolter has indicated that *rpoS* is not always beneficial and that mutations in *rpoS* can be a distinct advantage under certain circumstances (reviewed in Finkel et al., 2000). Studies on long-term stationary phase survival found that certain mutants exhibited a growth advantage in stationary phase (GASP) phenotype and were capable of out competing parental wild type strains (Zambrano et al., 1993). The first GASP-conferring mutation described was in an allele of *rpoS* (*rpoS*819) which had a small duplication near the 3' end of the gene that resulted in a protein with the last four amino acids replaced with thirty-nine new ones (Zambrano et al., 1993). In addition, GASP alleles have been isolated from aged *E. coli* cultures that

include missense and frameshift mutations and clinical and environmental isolates show similar allelic variation (Jishage and Ishihama, 1997), (Waterman and Small, 1996). Some mutations are known to affect protein stability, but for most mutant alleles the effect is undetermined. However, all mutations are thought to share partial loss of function in their ability to activate downstream genes. It is known that a full null mutation does not confer a GASP phenotype compared with the parental strain and so it is thought that some degree of *rpoS* function is still required (Zambrano et al., 1993).

The GASP phenotype can also result from mutations in other genes for example, *lrp* (Zinser and Kolter, 2000) and in genes that confer enhanced amino acid catabolism, such as *sgaA*, *B* and *C* (Zinser and Kolter, 1999). It has been proposed that these mutations are beneficial to the cell as GASP mutants are able to grow and even take over populations during stationary phase because they grow faster than their parental cells on the nutrients released from dead cells (Finkel et al., 2000). The fact that *rpoS* is hypervariable in nature and that partial loss of function is extremely common suggests that it is not necessary or beneficial for survival under all circumstances.

1.1.3 Promoter specificity

Even though σ^S enables a different set of genes to be expressed under stress conditions it is not strictly an alternative sigma factor as it does not use distinctly different promoter sequences to those of σ^{70} (Becker and Hengge-Aronis, 2001). The consensus sequence for σ^S seems to be extremely similar to that of σ^{70} as promoters that are transcribed by σ^S containing holoenzyme *in vivo* can also be expressed by σ^{70} mediated transcription *in vitro* (Nguyen et al., 1993; Tanaka et al., 1995). A number

of theories have been proposed to explain the selectivity of σ^S containing holoenzyme *in vivo*. Trehalose has been implicated in correct holoenzyme formation of RNA polymerase and σ^S promoter selectivity (Kusano and Ishihama, 1997) although this is now disputed (Germer et al., 1998; Kusano and Ishihama, 1997).

In vitro research has determined certain conditions that interfere with σ^{70} mediated transcription and thus selectively favour the expression of σ^S dependant genes. These conditions include: high salt, (Nguyen and Burgess, 1997), reduced negative supercoiling (Kusano et al., 1996) and the presence of additional regulatory proteins that bind in the promoter region (Hengge-Aronis, 1999). The last of these mechanisms is thought to enable high promoter specificity and examples of proteins that may be involved in this mechanism include H-NS, IHF, Lrp and Fis (Hengge-Aronis, 1999). Many are histone-like proteins, capable of binding DNA. However, the interaction of these global regulators with *rpoS*-regulated genes in complex. Often several factors act together to control single σ^S-dependant promoters and they may form complex nucleoprotein structures. Frequently these regulators positively control certain *rpoS*-dependant genes whilst negatively influencing others (Hengge-Aronis, 1999). For example, the *csiD* (carbon starvation inducible D) promoter is regulated positively by cAMP-CRP and lrp (Marschall et al 1998) whereas, *osmY* is repressed by cAMP-CRP, lrp and IHF (Lange et al., 1993). In a similar manner, H-NS directly controls many σ^s-regulated genes but represses many others (Hengge-Aronis, 1999).

H-NS is a DNA-binding protein that has been shown to regulate for the expression of several genes. H-NS is present at low levels during exponential growth but during

stationary phase, σ^S independent transcription of H-NS increases 10-fold. It has been proposed that H-NS DNA-binding plays a role in the promoter selectivity of σ^S and σ^{70} (Hengge-Aronis, 1999). At certain concentrations, H-NS has specificity for curved DNA and many σ^S promoters have innate curvature. It is thought the H-NS may stabilise these promoters in a conformation that prevents σ^{70} binding to them (Huisman et al., 1996).

There has been some controversy over the role of inorganic polyphosphate in promoter selectivity. It had been suggested that under conditions of high salt σ^{70} is inhibited and σ^S stimulated by inorganic polyphosphate (Kusano and Ishihama, 1997). However, this has been disputed by additional studies that have found that long chain polyphosphate has an inhibitory role in σ^S promoter selectivity. It was suggested that this could be due to the *in vitro* conditions used, specifically by the stripping of putative polyphosphate binding proteins from the core polymerase during the course of isolation from crude cell extracts (Shiba et al., 1997). The role of inorganic polyphosphate, if any, in promoter specificity has yet to be elucidated.

A number of σ^S-dependent promoters have been examined in detail and putative consensus sequences have been derived (summarised in Becker and Hengge-Aronis, 2001). σ^S dependant promoters generally have weak or absent –35 regions. This lack of a –35 region means that promoter activity is actually reduced for σ^S compared with σ^{70} holoenzyme as binding is weak and consequently transcriptional initiation is less effective. σ^S selective promoters often feature a TC motif at the –14/-13 positions and

a TATACT –10 hexamer. In addition, *in vivo* studies have found σ^S-dependent promoters show a preference for the sequence downstream from –10 to be AT rich.

1.1.4 Regulation of *rpoS*

The regulation of *rpoS* is complex and cellular levels of σ^S are controlled at the levels of transcription, translation and proteolysis (Lange and Hengge-Aronis, 1994; Loewen et al., 1993), reviewed in (Hengge-Aronis, 1999), summarised in fig. 1-1, fig. 1-2)

1.1.4.1 Transcriptional regulation of σ^S

Transcription of *rpoS* increases when cells grown in complex media enter stationary phase with a gradual change in growth rate. However, there is no increase in *rpoS*

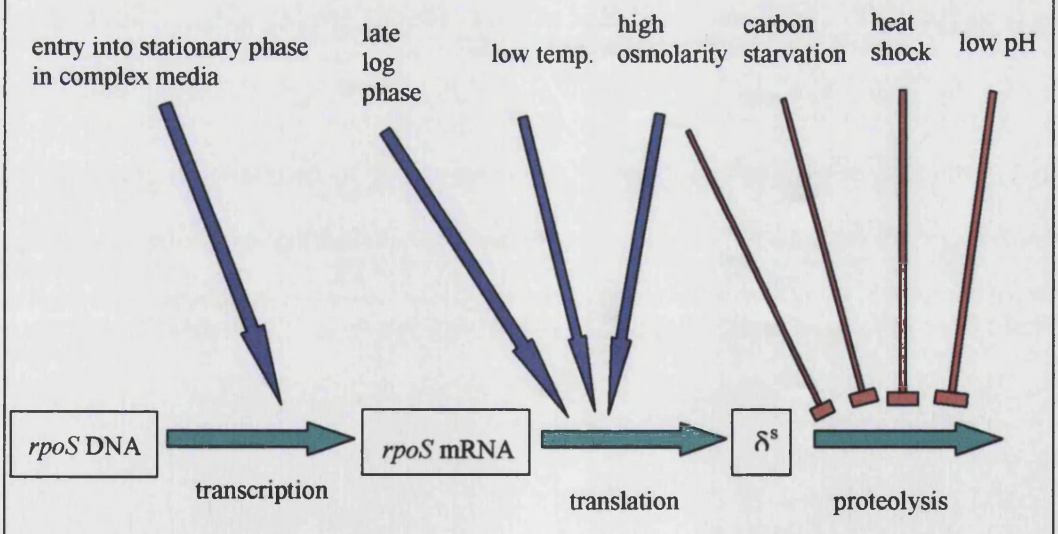


Fig.1-1. Various environmental stresses differentially effect *rpoS* transcription, translation and proteolysis. Blue arrows indicate positive regulation of the respective processes, red 'arrows' represent stabilisation of δ^s and/or inhibition of proteolysis. Adapted from Muffler *et al*, 1997.

mRNA if cells enter stationary phase in minimal medium (Lange and Hengge-Aronis, 1994). A number of small molecules have been found to regulate transcription positively (summarised in fig.1-2). The alarmone ppGpp influences expression and is thought to do so by positively effecting transcriptional elongation (Gentry et al., 1993). Inorganic polyphosphate and homoserine lactone are also thought to play a positive role in transcription but the exact mechanism is unknown (Shiba et al., 1997), (Huisman and Kolter, 1994). The only regulatory protein that has a characterised role in transcription is cAMP-CRP which has an inhibitory effect (Lange and Hengge-Aronis, 1994). A putative biding site for this molecule is upstream of the *rpoS* promoter located within the *nlpD* gene (Hengge-Aronis, 2000). More recently, it has been suggested that BarA, a probable two-component regulator is required for exponential phase induction of *rpoS* and it is thought that this may act at the transcriptional level (Mukhopadhyay et al., 2000).

1.1.4.2 Translational regulation of σ^S

Translation of *rpoS* mRNA increases with shift to high osmolarity (Muffler et al., 1996) and temperature downshift (Sledjeski et al., 1996) and during late exponential phase (Lange and Hengge-Aronis, 1994). It has often been speculated that the translational regulation of *rpoS* results from changes in conformation of the mRNA secondary structure (Wang and Kim, 2000; Hengge-Aronis, 1999) and the exact mechanism for this control has now been elucidated. It is known that a long leader region required for transcriptional regulation precedes the mRNA coding region for RpoS (Lange et al., 1995). This region is capable of forming a hairpin stem-loop structure that blocks the ribosome binding site and prevents translational initiation (Brown and Elliot, 1996).

Translation is increased by the expression of a small RNA, *dsrA* in response to low temperature (Sledjeski et al., 1996). *DsrA* interacts with *rpoS* mRNA, preventing the formation of the hairpin and thus enabling high levels of translation (Majdalani et al., 1998). In a similar manner, an additional RNA *rprA* has been found to contribute to the regulation of *rpoS* translation (Majdalani et al., 2001). A number of proteins are also involved in the regulation of *rpoS* translation. The HF-I protein (also referred to as Hfq) activates translation under appropriate conditions. HF-I binds *rpoS* mRNA and induces conformational changes that disrupt the stem-loop structure (Muffler et al., 1996a). Recently, the HU protein has been found to be regulate translation positively. HU is a member of the family of DNA architectural proteins and a major component of the bacterial nucleoid. It increases translational efficiency by an unknown mechanism (Balandina et al., 2001).

σ^S translation is also subject to negative regulation by H-NS, the small metabolic product UDP-glucose (Hengge-Aronis et al., 1995) and the small RNA *oxyS* (Altuvia et al., 1997). *OxyS* is thought to interfere with translation by the formation of a translationally incomplete complex with HF-1 and *rpoS* mRNA. As *oxyS* is induced in response to hydrogen peroxide it has been proposed that this mechanism of translational inhibition prevents the unnecessary induction of σ^S when the oxidative stress response alone would suffice.

1.1.4.3 Post-translational regulation of σ^S

Rapid alterations in cellular σ^S levels are generally as a result of decreased proteolysis by ClpXP protease (Wang and Kim, 2000; Hengge-Aronis, 1999). Under non-stress conditions RpoS is highly unstable with a half-life of approximately 2 minutes

(Muffler et al., 1996; Lange and Hengge-Aronis, 1994; Muffler et al., 1997). In response to stresses such as starvation, shift to low pH, high temperature and high osmolarity, proteolysis is inhibited and the half-life can increase to as much as 50 minutes (Hengge-Aronis, 1996). However, it is important to note that studies involving starvation have generally relied upon the use of carbon limitation and that this has been carried out in poorly defined minimal media (modified M9) (Lange and Hengge-Aronis, 1994; Zgurskaya et al., 1997). Under these conditions it is feasible that σ^S stabilisation results from the combined effects of carbon-starvation, pH drop and iron limitation.

The mechanisms involved in σ^S turnover have been well characterised. For normal turnover of σ^S, 3 factors are essential: ClpXP protease (Schweder et al., 1996), the response regulator RssB (Bearson et al., 1996; Muffler et al., 1996b; Pratt and Silhavy, 1996) and the cis-acting turnover element within σ^S (Schweder et al., 1996; Muffler et al., 1996). ClpX acts as the regulatory component of ClpXP that is believed to unfold RpoS and eventually feed it into ClpP the proteolytic component (Zhou et al., 2001). RssB is also referred to as SprE and acts as a direct recognition factor that interacts with the turnover element in σ^S in a manner dependant on the phosphorylation of the RssB receiver domain (Becker et al., 1999). It has recently been shown that RssB also plays a catalytic role in the delivery of *rpoS* to ClpX (Zhou et al., 2001). Interestingly, it has now been shown that RssB may bind σ^S without leading to direct proteolysis thus, interfering with σ^S activity (Becker et al., 2000). Under these circumstances, RssB can be said to be acting as an antisigma factor to RpoS. Another intriguing point about RssB is that the binding site within RpoS (the turnover element) is the same area that is important for recognition of the extended –

10 region of promoters (Becker et al., 2000). The turnover element consists of a small patch of amino acids (with lysine 173 at its core) that is not present in σ^{70} and is located just downstream of the promoter recognition region 2.4 (Hengge-Aronis, 1999). Therefore, it is possible that the binding of RssB to σ^{S} could influence promoter specificity.

An additional protein has been implicated as having a role in σ^{S} proteolysis. The chaperone DnaK is known to promote structural rearrangements in proteins and is thought to play a role in stabilising σ^{S} and increasing half-life (Rockabrand et al., 1998).

It has been suggested that an additional level of *rpoS* regulation exists via the protein Crl. It has been proposed that Crl acts upstream or in concert with *rpoS* to regulate positively the *rpoS* regulon (Pratt and Silhavy, 1998). It is thought that Crl may associate with or covalently modify RpoS or σ^{S} holoenzyme therefore, influencing activity.

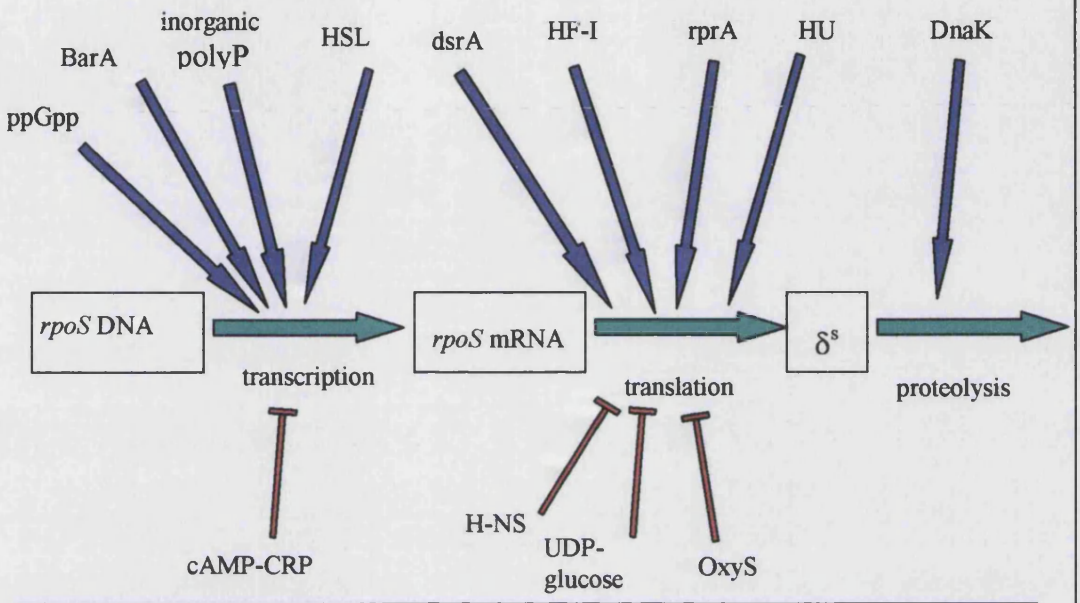


Fig. 1-2. The effect of regulatory proteins and small RNAs on *rpoS* transcription, translation and proteolysis. Blue arrows indicate positive regulation of the respective processes, red 'arrows' represent stabilisation of δ^s and / or inhibition of proteolysis.

1.1.4.4 Influence of cell density on σ^S

In addition to the interplay with other regulatory networks (notably ppGpp and inorganic phosphate), cell density has also been included among possible factors affecting σ^S levels (Hengge-Aronis, 1996). Much evidence for density dependant effects has been indirect (Huisman and Kolter, 1994) or has relied on the use of spent culture medium (Sitnikov et al., 1996). However, recently its influence on

expression has been demonstrated in a chemostat model where it was found that there was an eight-fold increase in RpoS levels between bacteria grown at 10^8 and 10^9 cells/ml (Liu et al., 2000). However, to date the separate influences of growth rate, cell density and nutrient limitation on *rpoS* have not been elucidated.

1.2 STRINGENT RESPONSE

The stringent response is a physiological adaptation to nutritional stress that primarily results in the inhibition of stable RNA synthesis (reviewed in Chatterji and Kumar Ojha, 2001; Cashel et al., 1996). The effector is the alarmone nucleotide (p)ppGpp. Amino acid starvation results in a specific interaction between uncharged tRNA and the ribosome. This leads to the activation of ribosome-bound ppGpp synthetase I (PSI), encoded by *relA*. PSI catalyses the synthesis of the pentaphosphate form of the nucleotide (pppGpp) that is subsequently degraded to ppGpp by the enzyme pppGpp-5'-phosphohydrolase. An additional pathway for ppGpp synthesis exists and is utilised if the cell is starved for carbon, inorganic phosphate or phospholipids. This second pathway is dependent on the bifunctional protein SpoT. SpoT possesses dual activity for the synthesis and degradation of ppGpp. Synthetic activity is represented by ppGpp synthetase II (PSII) and hydrolytic activity by ppGpp hydrolase (Gentry and Cashel, 1996). Interestingly, it has been shown that during glucose starvation accumulation of ppGpp is predominantly a result of the inhibition of SpoT hydrolase activity (Murray and Bremer, 1996).

ppGpp initiates global changes in gene expression, directly connecting nutritional stress to adaptive responses. The effect of the stringent response is not limited to the rapid cessation of stable RNA synthesis (summarised in fig. 1-3). There is also a swift shutdown of macromolecular processes that would be wasteful to cells that are nutrient limited and in a non-growing state (Svitil et al., 1993). There is a decrease in the synthesis of peptidoglycan, phospholipids and ribosomal proteins (Cashel et al., 1996). In addition, DNA replication is inhibited, as this too is unnecessary in non-growing cells. Mutants that fail to elicit the stringent response show significantly reduced viability during stasis (Nyström, 1994). This reduced viability is thought to be due to a combination of wasteful macromolecular activity, lack of certain protective / repair mechanisms and the absence of σ^S activity and associated resistance mechanisms (Cashel et al., 1996).

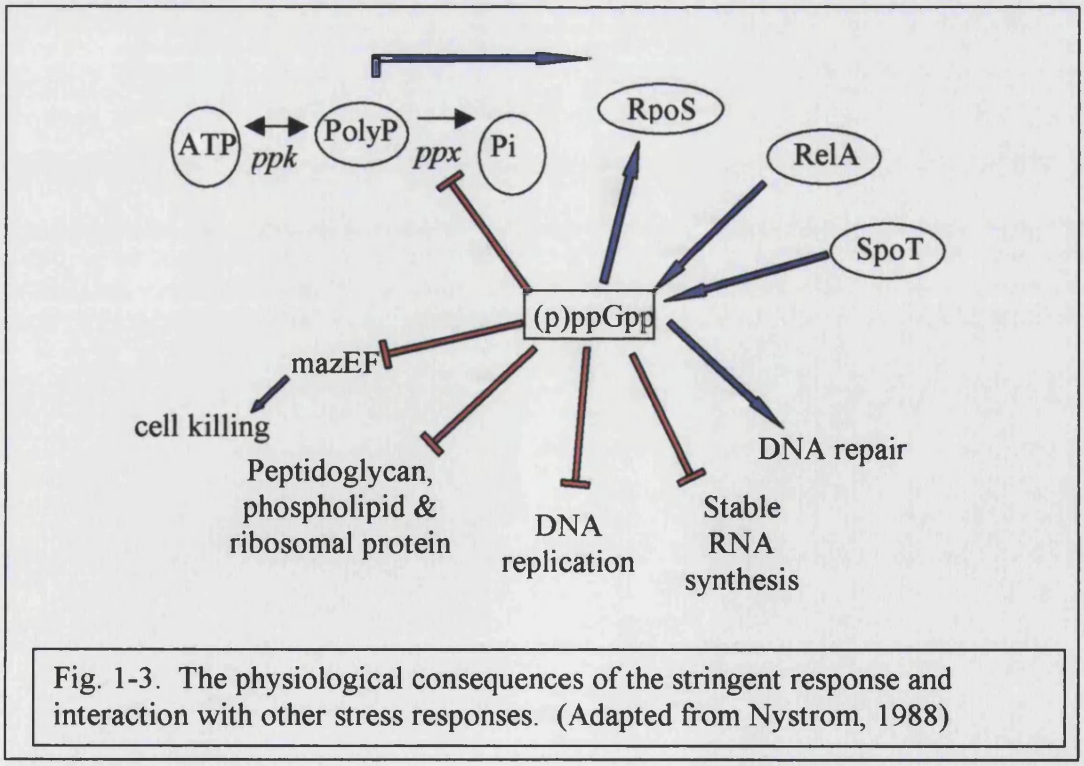


Fig. 1-3. The physiological consequences of the stringent response and interaction with other stress responses. (Adapted from Nystrom, 1988)

Significantly, ppGpp is essential for the activity of σ^S (Gentry et al., 1993). It is mandatory for the expression of σ^S regulated promoters and causes conformational changes in RNA polymerase that assist in promoter selectivity (Kvint et al., 2000). RpoS levels are also modulated by the increased cellular levels of inorganic polyphosphate that accumulate during the stringent response (Shiba et al., 1997; Kuroda et al., 1997). However, the role of inorganic polyphosphate in stress responses will be discussed separately. Interestingly, in a manner analogous to the global changes in gene expression seen by ppGpp mediated induction of *rpoS*, it is also crucial for early spore development in *Myxococcus xanthus* (Harris et al., 1998).

The stringent response also has a role in protection and repair mechanisms. ppGpp participates in the DNA repair pathway initiated by UV light (McGlynn and Lloyd G, 2000). UV-induced lesions in DNA stall the elongation complex of RNA polymerase at the site of damage. ppGpp mediates early dissociation of the elongation complex and therefore, facilitates the regression of the elongation fork, the repair of lesions and reinitiation of the elongation fork by a RecG-dependent pathway. Interestingly, *recG* and *spoT* are present on the same operon. The stringent control network also has an important role in maintaining translational fidelity during stasis (Nyström, 1994). In the absence of ppGpp the combination of excess mRNA and uncharged tRNA results in translational error. In ppGpp null mutants the translational machinery is severely affected and mistranslations and frameshifts result frequently (Nyström, 1994).

The stringent response has been found to regulate positively the production of secondary metabolites in a number of organisms. For example, the production of antibiotics in many species of *Streptomyces* is ppGpp-dependent and in an analogous

manner, so is the production of colicin K in *E. coli* (Chakraburtty and Bibb, 1997; Kuhar and Zgur-Bertok, 1999). The stringent response is linked to the pathogenesis of a number of organisms but this has not been widely studied in *E. coli*, *S. typhimurium* or *P. aeruginosa*. In *L. pneumophila* many stationary phase virulence factors are dependent on ppGpp and in *Mycobacterium tuberculosis* the stringent response has been implicated in long term survival under anaerobic and starvation conditions (Hammer and Swanson, 1999; Primm et al., 2000).

Paradoxically, given the role of the stringent response in the survival of cells during stationary phase and conditions of nutrient starvation, it also has a role in 'cell suicide' (summarised in Nyström, 1998). Intriguing results have indicated that *E. coli* possess genetic systems that are responsible for programmed cell death (Aizenman et al., 1996). Chromosomally encoded modules have been identified that consist of two genes encoding a stable toxin and an unstable antidote. Cells are 'addicted to', that is, entirely reliant on, the antidote to prevent killing by the toxin. The antidote is encoded by *mazE*, and the toxin by *mazF* and the two together form an operon with *relA*. MazEF is negatively regulated by ppGpp. Presumably, killing by *mazEF* would occur under conditions of the stringent response, which would block *mazEF* transcription and enable the more stable toxin to express lethal function while the unstable antidote is degraded. It has been suggested that this system could serve as a mechanism for 'altruistic cell death' during starvation where lysis of some cells would provide nutrients for others (Nyström, 1998). It has not been discovered how this system would respond to a *spoT*-mediated stringent response, that is, does this system function only under *relA*-mediated stringent control in response to amino acid

starvation? It has been proposed that some type of density-dependent control must influence the *mazEF* system to prevent 'programmed cell death' at low cell density.

Recently, a link between the stringent response and density-dependent quorum sensing has been shown for *P. aeruginosa* (Van Delden et al., 2001). It was found that both *rhlR* and *lasR* gene expression, and autoinducer synthesis were prematurely activated during the stringent response (by overexpression of RelA) in a manner independent of *rpoS*. No relationship between the stringent response and density dependant events has been found in *E. coli*.

1.3 INORGANIC POLYPHOSPHATE

Inorganic polyphosphate (poly P) is a linear polymer of tens to hundreds of orthophosphate residues (Pi) linked by high-energy phosphoanhydride bonds. In *E.coli* poly P is generally present at concentrations ranging from 0.1 to 50 mM but this may be amplified as much as one thousand-fold in response to stress (reviewed in (Kornberg et al., 1999). Poly P is of extreme interest as it is found in every living cell, but until recently it had no known function and was dismissed as a 'molecular fossil'. It is now known that it has a wide range of functions depending on the species and cellular location. For example, poly P can act as a substitute for ATP in kinase reactions, serve as a reservoir of Pi, chelate many metals, buffer against alkali, contribute to the capsule of some bacterial species and it is required for bacterial competence and for mRNA processing and degradation. More recently, it has been shown to play a crucial role in virulence and even biofilm formation as it is essential for bacterial motility (Rashid and Kornberg, 2000; Rashid et al., 1999). Perhaps most

importantly, it also plays a regulatory role in physiological adjustments to growth and stress (Kornberg et al., 1999).

In *E.coli* poly P is produced by a polyphosphate kinase. This converts the terminal phosphate of ATP to give poly P and can also catalyse the reverse reaction, converting poly P to ATP. It also possesses the ability to convert other nucleotides, especially GDP to GTP. Polyphosphate kinase is encoded by the gene *ppk* that constitutes an operon together with *ppx* (Kornberg et al., 1999) (shown in fig. 1-3). The promoter region contains two putative PhoB boxes that respond to low phosphate levels in the medium. *ppx* encodes an exopolyphosphatase and along with *ppk*, removes poly P to maintain a dynamic balance of the cellular poly P level. *ppk* levels are known to be highest upon entry into stationary phase whereas *ppx* levels are lowest. It is not known how the activity of the two genes is differentiated or whether there is post-transcriptional control. Interestingly, it is known that during rapid poly P accumulation there are only minimal changes in gene expression (Kornberg et al., 1999).

In response to some stresses a rapid one hundred-fold increase in poly P occurs that can last for minutes or even hours (Kornberg et al., 1999). Stresses that have been found to cause poly P accumulation include amino acid starvation, nitrogen depletion, phosphate depletion, nutrient downshift and high salt. Interestingly, carbon-depletion and exposure to acid had no effect on poly P levels (Kornberg et al., 1999).

Depletion of phosphate leads to rapid poly P accumulation (up to 20 mM). Low levels of inorganic phosphate are sensed by a phosphate regulon (Pho) that includes

the *pst* pathway for Pi uptake and the *phoB* response regulator that activates numerous genes involved in phosphate uptake and assimilation (Wanner, 1996). Poly P accumulation is dependent on PhoB and is abolished in PhoB null mutant strains (Rao et al., 1998). It also requires the high levels of (p)ppGpp associated with the stringent response (Rao et al., 1998).

So it can be seen that under some circumstances the stringent response is required for increases in cellular poly P (Cashel et al., 1996; Kuroda et al., 1997). However, elevated (p)ppGpp levels are not always required or sufficient for poly P accumulation. For example, under conditions of carbon starvation and in PhoB null mutants there is accumulation of (p)ppGpp but no concurrent increases in poly P (Kornberg et al., 1999). When poly P does accumulate in response to (p)ppGpp it is thought that this is due to the inhibitory action of the alarmone on *ppx*. By inhibiting the breakdown of poly P by *ppx*, poly P has a greatly increased half-life. This could explain why the activities of *ppk* and *ppx* change only marginally during the rapid accumulation of poly P as they are subject to additional regulatory control (Kornberg et al., 1999).

Poly P is also accumulated in response to nitrogen (ammonia) depletion in minimal salts media. Increases in poly P are dependent on *glgG* (the sensor for ammonia assimilation) and *glnD* (the gene of the regulatory protein NtrC). Null mutants of either of these genes fail to accumulate poly P. Surprisingly, mutants in the nitrogen starvation associated sigma factor σ^{54} (encoded by *rpoN*) show normal accumulation of poly P (Ault-Riché et al., 1998). Poly P is also accumulated in response to

nutritional downshift, that is, where exponential cells from rich media are transferred to minimal media (Ault-Riché et al., 1998).

Poly P has also been shown to be significant for some *rpoS*-regulated events. Cells deficient in poly P fail to express some *rpoS*-regulated genes and show low stationary phase viability (Rao and Kornberg, 1996). Mutants lacking *ppk* exhibit between ten- and a hundred-fold increased susceptibility to heat, oxidative stress and osmotic shock (Shiba et al., 1997; Rao and Kornberg, 1996; Crooke et al., 1994). This increased sensitivity is known to be a result of *rpoS* effects as complementation with either *ppk* or extra copies of *rpoS* restored resistance (Shiba et al., 1997; Rao and Kornberg, 1996). Importantly, poly P is also required for *rpoS* expression. Cells that were deficient in poly P as a result of the addition of the potent yeast exopolyphosphatase showed no elevation of *rpoS* in response to starvation (Shiba et al., 1997). Poly P is also thought to play a role in σ^S promoter specificity (discussed earlier).

Interestingly, poly P is also important for the regulation of stress responses that act independently of *rpoS*. Recently, it was shown that poly P regulates the expression of a number of SOS response genes, particularly *recA* (Tsutsumi et al., 2000)

1.4 Quorum Sensing

The perception and processing of chemical information from the environment forms a central role in the regulation of adaptive stress responses. Chemical changes sensed in the environment may result from the metabolic processes of bacteria themselves, specifically the production of diffusible signals for cell-to-cell communication within the bacterial population. Sensing and responding to diffusible signals (called autoinducers) is termed quorum sensing (reviewed in Bassler, 1999 and Withers et al., 2001). The concentration of autoinducers reflects cell density and a threshold level indicates that the population is 'quorate' that is, capable of making co-ordinated changes in gene expression.

Engebrecht and Silverman described the first quorum sensing mechanism in the bioluminescent marine organism *Vibrio fischeri* (Engebrecht and Silverman, 1984). This mechanism relies on the production of acylated homoserine lactones (AHLs) that are produced and accumulate in the external environment. When a threshold level is reached a signal transduction cascade is initiated that results in the production of luciferase. Two regulatory protein, LuxI and LuxR control light production in *V. fischeri*. LuxI is an autoinducer synthase and LuxR is a transcriptional activator protein that is capable of binding autoinducers thus promoting transcription of the luciferase structural operon *luxCDABE* (Engebrecht and Silverman, 1984; Engebrecht and Silverman, 1987).

This simple, signal-response mechanism is widespread in over thirty species of Gram negative bacteria, excluding *E. coli*. Although there is variation in the genes activated

by this type of quorum sensing all of these systems have in common an AHL autoinducer whose synthesis is dependent on a LuxI homolog and a LuxR homolog that is responsible for detection of autoinducer and activation of the appropriate genes (Bassler, 1999). It is interesting to note that in *P. aeruginosa* this system has been closely linked to the general stress response. Recently, there has been some debate over the hierarchy of regulation and whether quorum sensing regulates *rpoS* or vice versa (Latifi et al., 1996). It now appears that *rpoS* itself regulates quorum sensing (Whiteley and Parsek, 2000).

Further research on the free-living relative of *V. fischeri, V. harveyi* has identified a second, non-AHL quorum sensing pathway (Bassler et al., 1994). This system is more reminiscent of the two component signal transduction networks that are utilised for quorum sensing in Gram positive bacteria and production of this second (AI-2) molecule is dependent on the LuxS gene (Surette et al., 1999; Surette and Bassler, 1998). AI-2 is recognised by a cognate sensor kinase protein named LuxQ and it is thought that a periplasmic binding protein called LuxP interacts with LuxQ to recognise AI-2. Sensory information is transduced by phosphorylation and dephosphorylation to a signal integrator protein called LuxU (shared with the AHL circuit), which subsequently conveys the signal to the response regulator protein LuxO. LuxO controls transcription of the luciferase structural operon (Bassler, 1999) (summarised in fig. 1-4). Highly conserved *luxS* homologs are found in both Gram positive and Gram negative bacteria, including *E. coli* and *S. typhimurium*. It has been suggested that AI-2 is used for both inter- and intra-species communication as *V. harveyi* are capable of responding to AI-2 from other species (Surette and Bassler, 1998). The ability of *V. harveyi* to respond to AI-2 has been central to the progression

of research into LuxS-regulated quorum sensing. Surette and co-workers constructed reporter strains of *V. harveyi* that are deficient in AHLs and respond specifically to AI-2 (Surette and Bassler, 1998). These strains have been invaluable for the detection and study of AI-2 in other species.

In *E. coli* AI-2 activity was found to be maximal in late exponential phase and decreased as cells entered stationary phase (Surette et al., 1999). AI-2 is induced in response to a number of environmental cues including; high osmolarity and low pH (Surette and Bassler, 1999), the presence of preferred carbon sources (Surette and Bassler, 1998; DeLisa et al., 2001) and Fe^{3+} (DeLisa et al., 2001). Decreased activity

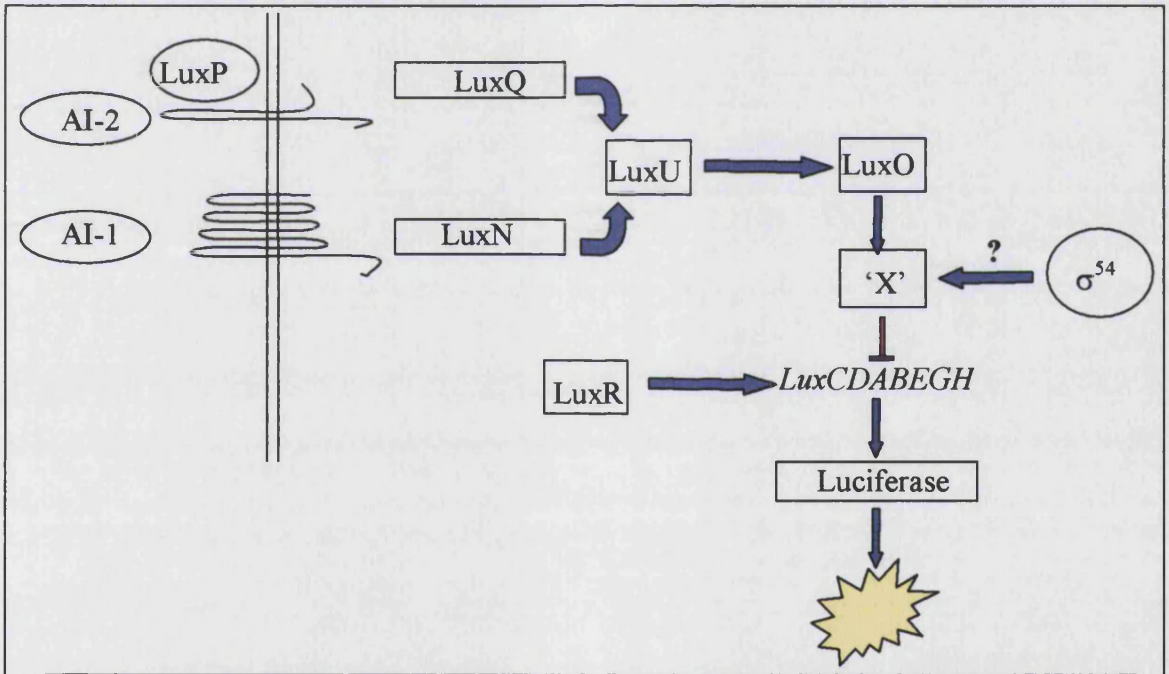


Fig. 1-4. Model for quorum sensing in *Vibrio harveyi* showing the two parallel quorum sensing circuits. A two-component signal transduction system relays autoinducer detection to the luciferase structural operon, leading to light production. (Adapted from Schauder et al, 2001)

was found in response to anaerobiosis and amino acid starvation in chemostat populations (DeLisa et al., 2001).

A putative structure and the biosynthetic pathway for AI-2 have now been determined (Schauder et al., 2001). The pathway for AI-2 production was previously thought to be a pathway for salvaging adenine and homocysteine. AI-2 is produced from S-adenosylmethionine (SAM), which is converted to S-adenosylhomocysteine (SAH) by the action of numerous methyltransferases. Subsequently, SAH is converted to S-ribosylhomocysteine (SRH) by Pft nucleosidase, liberating adenine. LuxS, as expected is the AI-2 synthase and acts on SRH to produce an unstable compound that spontaneously cyclizes to AI-2, thought to be a furanone (Schauder et al., 2001). This is interesting as these molecules have previously been implicated as signalling molecules in both prokaryotes and eukaryotes. Furanones are structurally related to AHLs and have been shown to inhibit AHL-mediated swarming motility in *Serratia liquefaciens* by inactivation of the LuxR transcriptional activator (Manefield et al., 1999). Therefore, the possibility arises that AI-2 could antagonise AHL-mediated quorum sensing.

Recently, microarray based approaches have shown that expression of a large number of genes is influenced by A1-2. One study found that 242 genes (approximately 5.6% of the *E. coli* genome) exhibited significant transcript changes in response to AI-2 (DeLisa et al., 2001) whereas another found that as many as 404 genes were influenced by AI-2 (Sperandio et al., 2001). Clearly, AI-2 mediated quorum sensing must have global effects on gene expression.

Quorum sensing has been linked to virulence in *E. coli*. It has been found to have a regulatory effect on the locus of enterocyte effacemant (LEE operon) of O157:H7 (Sperandio et al., 1999). This pathogenicity island is important for the intimate attachment of bacteria to the epithelial cell via the type III-secretion system. It is thought that the role of LuxS in type III-secretion may be modulatory rather than regulatory as this system is still functional in DH5α (a strain deficient in LuxS) (Sperandio et al., 1999; Gruenheid and Finlay, 2000). It has been suggested that *in vivo* studies are necessary to clarify the role of quorum sensing in regulation of the LEE operon (Gruenheid and Finlay, 2000). Quorum sensing has also been implicated in the production of Shiga toxins in enteropathogenic *E. coli* (Sperandio et al., 2001). It has been proposed that quorum sensing may explain the low infectious dose of *E. coli* O157 (Sperandio et al., 1999). It is feasible that the intraspecific nature of LuxS quorum sensing enables communication between O157 and the resident, non-pathogenic gut flora.

More recently quorum sensing has been implicated in regulating the activity of *sdiA*, a LuxR-type transcriptional activator of the cell division operon *ftsQAZ* (Sitnikov et al., 1996; DeLisa et al., 2001a). However, the evidence for a link between AI-2 and SdiA is conflicting. Recently evidence from reporter constructs (Surette and Bassler, 1999) and microarray analysis (DeLisa et al., 2001b) has indicated that AI-2 does not significantly effect SdiA and that SdiA is not the transcriptional activator of LuxS-mediated events.

It is feasible that SdiA may be a transcriptional activator of another, as yet undiscovered quorum sensing pathway. SdiA has a role in pathogenesis of

enterohaemorrhagic *E. coli*. Culture supernatant components were found to bind the N-terminal region of SdiA and virulence factors were controlled in a quorum sensing fashion (Kanamaru et al., 2000). More recently microarray analysis of SdiA amplification has shown that it influences the transcription of nearly 150 genes, most of which are related to motility and chemotaxis (Wei et al., 2001). Interestingly, the *acrABDEF* efflux genes (discussed later) are significantly upregulated by SdiA and several genes involved in DNA repair and replication are moderately influenced (Wei et al., 2001).

There are some indications of links between AI-2 and stress responses. Significant induction of genes associated with the nitrogen starvation sigma factor σ^{54} has been reported (DeLisa et al., 2001). Both *ygeV*, a putative σ^{54}-dependent transcriptional activator and *yhbH*, a σ^{54} modulating protein were significantly induced in response to overexpression of AI-2 (DeLisa et al., 2001). It has been postulated that *E. coli* may employ σ^{54} during quorum sensing in a manner analogous to *V. harveyi* (fig. 1-4). Links have also been found between the SOS response to DNA damage and AI-2. Genes such as *recA*, *uvrA* and *sulS* were upregulated in a wild type compared with a LuxS null mutant strain (Sperandio et al., 2001). Perhaps unsurprisingly, (bearing in mind that AI-2 is degraded upon entry into stationary phase) no links have been found between *rpoS* and AI-2.

1.5 THE MARRAB OPERON AND ACRAB-TOLC MULTIPLE DRUG EFFLUX PUMP

1.5.1 The *marRAB* global regulatory system

Evolution has tailored bacteria with an immense capability to survive within a toxic environment. The *marRAB* global regulatory system and subsequent downstream events are thought to have evolved to enable bacteria to survive exposure to noxious natural agents such as the plant compounds plumbagin and the bile salt rich environment of the mammalian gastrointestinal tract (Kawamura-Sato et al., 1999; Miller and Sulavik, 1996; Thanassi et al., 1997). However, *marRAB* is also responsible for conferring resistance to a wide range of toxic compounds including multiple antibiotics, organic solvents, oxidising agents and household disinfectants (Alekshun and Levy, 1998). The *marRAB* system was discovered by exposing *E. coli* to tetracycline or chloramphenicol at levels in excess of the MIC. The resulting mutants were found to exhibit resistance to a broad range of antibiotics including β-lactams, rifampicin and nalidixic acid. This phenotype was designated mar (multiple antibiotic resistant) and was found to be associated with decreased accumulation (George and Levy, 1983). The three-gene operon *marRAB* comprises of the negative regulator *marR*, *marA* the positive effector that is epistatic to *marR* in promoting resistance and *marB* whose function in unknown (Gambino et al., 1993).

Salicylate has been shown to stimulate transcription of the operon (Cohen et al., 1993) and is even more effective in this respect than either tetracycline or chloramphenicol (Cohen et al., 1993; Hachler et al., 1991). Salicylate acts by antagonising the repressor activity of *marR* by directly binding to it so that it no longer represses the *marRAB* operon (Martin and Rosner, 1995)

Activation of the *marRAB* regulatory system changes the expression of several unlinked chromosomal target genes. The outer membrane porin OmpF is down regulated by the production of *micF* antisense RNA (Cohen et al., 1988). Genes positively regulated by *marRAB* include the multiple drug efflux system *acrAB* (Ma et al., 1995).

In addition to MarA, overexpression of SoxS and Rob (other proteins within the AraC / XylS family) leads to a mar phenotype. The *soxRS* regulon plays an important role in resistance to oxidative stress (reviewed in Stortz and Zheng, 2000). Rob was identified by its ability to bind DNA near the bacterial origin of replication (Skarstad et al., 1993). The SoxS, MarA and Rob transcriptional activators bind many, if not all, of the same promoters. Many of these promoters have asymmetric "soxbox" / "marbox" regions that have been defined by extensive promoter studies (Stortz and Zheng, 2000). Recent work indicates that some promoter discrimination between SoxS and MarA is due to slight differences in binding to the individual mar / soxbox regions (Stortz and Zheng, 2000). Each of the transcriptional activators is functional in the absence of the others and the reasons for the extensive overlap between the *soxRS*, *marA* and *rob* regulons is not understood. However, unless induced, the contribution of SoxS to the activation of MarA, and subsequently AcrAB-TolC, is negligible (Alekshun and Levy, 1998).

1.5.2 AcrAB efflux pump

Chromosomally encoded efflux systems play a central role in determining intrinsic levels of antibiotic resistance. Efflux systems can confer resistance to a broad range of structurally unrelated compounds. If specific mechanisms of antibiotic resistance

are absent then increased resistance to antibiotics can occur only by decreasing the ability of the drug to accumulate within the cell below the levels of growth inhibition. Drug accumulation may be reduced by deployment of efflux pumps that utilise energy from ATP hydrolysis or proton motive force (Lewis, 1994) or by the down regulation of surface porins through which drugs enter the cell (Nikaido, 1994). Efflux pumps are modulated in response to the environment and many are inducible by the substances they expel (Miller and Sulavik, 1996).

Most efflux pumps have a three component organisation with an inner membrane transporter, outer membrane channel and periplasmic accessory protein that enable efflux directly out of the cell bypassing the outer membrane barrier (Zgurskaya and Nikaido, 2000). The *acrAB* system comprises of *acrA* a membrane fusion protein (MFP) that acts as the periplasmic accessory protein, *acrB* an inner membrane transporter of the RND (resistance nodulation cell division) family (Zgurskaya and Nikaido, 1999). However, *acrAB* lacks the gene for the outer membrane component and instead recruits TolC, a multifunctional outer membrane channel to complete the tri-partite complex (Fralick, 1996) (summarised in fig. 1-5)

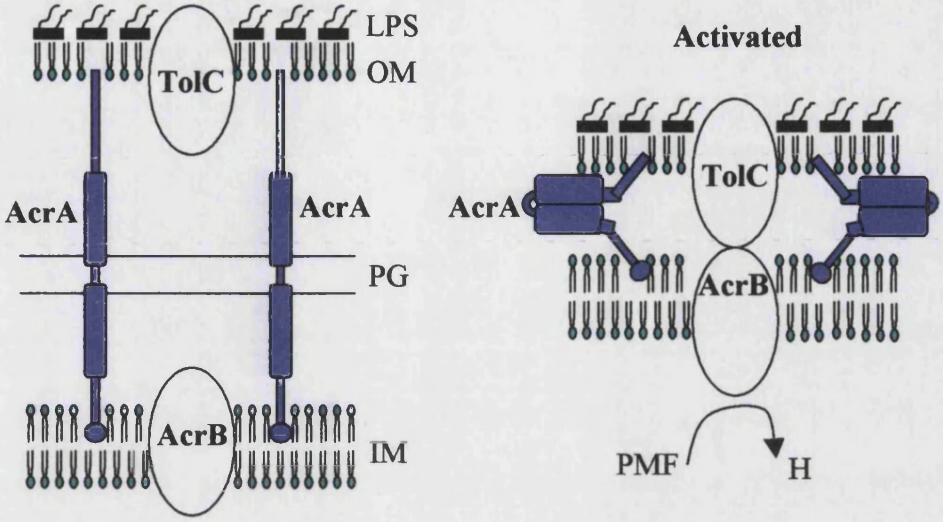


Fig. 1-5. The AcrAB-TolC tri-partite multidrug efflux pump. Upon activation, acrA links the outer (OM) and inner membranes (IM) closely together and enables drug efflux directly out of the cell (bypassing the periplasm and peptidoglycan, PG) by the use of proton motive force (PMF), liberating a hydrogen ion (H^+).

The *acrAB* efflux pump is an important target for MarA in the mar phenotype (Ma et al., 1996). An additional level of regulation of the operon is by the upstream and divergently transcribed *acrR* that encodes a repressor of *acrAB* (Ma et al., 1995). In addition to stimulation by MarA and SoxS, *acrAB* is also positively regulated by environmental stress including that of high salt, decanoate, ethanol and there is some indication that expression may be growth phase regulated (Ma et al., 1996). MarA or SoxS do not regulate induction of *acrAB* by environmental stress and interestingly, under these conditions *acrR* itself acts in a negative feedback loop to prevent excessive expression of *acrAB* that could be detrimental to the cell (Ma et al., 1995).

1.6 MECHANISMS OF BIOFILM RESISTANCE TO ANTIMICROBIAL AGENTS

Biofilms are sessile bacterial communities that live attached to each other and/or surfaces enclosed in an exopolysaccharide matrix. Biofilms can be of a single bacterial species but are commonly comprised of a mixed consortium (Costerton, 1995; Costerton et al., 1995). The biofilms that are present in our intestines, sewage treatment plants, bioremediation plants and used as biocontrol agents are largely beneficial (Geels and Schippers, 1983). However, biofilms are frequently detrimental, are found in the lungs of cystic fibrosis patients, in dental plaque, and create problems by the colonisation of indwelling medical devices, particularly catheters (Costerton et al., 1999). It has been estimated that biofilms are associated with 80 % of nosocomial infections where they significantly increase patient morbidity and mortality (Costerton and Stewart, 2001). Biofilms also cause significant problems in industry where they are associated with the biofouling of ships and the clogging of industrial pipes (Costerton et al., 1995).

Biofilm structure is highly variable and dependent on a number of factors, including the organism, the surface, the surrounding nutrient environment and the rate of flow of any aqueous interface (Costerton et al., 1995; Karthikeyen et al., 2000). Biofilms may vary in configuration, from sparse amorphous masses to highly complex, organised structures with mushroom-like cell stacks interspersed with water-filled channels (Costerton et al., 1999). These channels have been compared with circulatory systems, leading to the emerging view that biofilms can be considered analogous to primitive multicellular organisms (Costerton et al., 1999).

The most important and widely studied property of biofilms is their vastly increased resistance to antimicrobials. Compared with planktonic cells, biofilms are commonly 10-1000 times more resistant (Mah and O' Toole , 2001). The development of biofilm resistance is not well understood but a number of mechanisms are thought to act synergistically (reviewed in Mah and O' Toole, 2001; Lewis, 2001; Smith and Brown, 2001). It has been proposed that multiple mechanisms of resistance exist which vary in importance depending on the species of bacteria present and the antimicrobial agent being applied (Mah and O' Toole, 2001). These resistance mechanisms include the failure of antimicrobial agents to penetrate the biofilm, decreased growth rate, the general stress response, high cell density and induction of a 'biofilm phenotype'.

1.6.1 Failure of antimicrobials to penetrate the biofilm

Biofilms are characteristically enclosed within an exopolysaccharide matrix or glycocalyx that can restrict the diffusion of and bind antimicrobial agents (Stewart, 1996). The mechanism is thought to be most effective in protecting biofilms against large, host-produced molecules (lysozyme and complement). However, it is thought only to postpone the influx of small antimicrobial agents (Lewis, 2001). Delayed penetration may afford some protection against degradable antimicrobials as the antibiotic is rendered harmless before it permeates the biofilm. The diffusion barrier provided by the EPS may also be important for resistance to hydrogen peroxide (Elkins et al., 1999). Although the physical barrier of the EPS may contribute to biofilm resistance, a number of studies have indicated that this mechanism alone is not sufficient to explain the recalcitrance of biofilms to antimicrobial agents (Mah and O' Toole, 2001).

At a cellular level, the decreased influx of antimicrobial agents is often associated with concomitant increased efflux (Nikaido, 1998). As discussed previously, multidrug efflux pumps can enable the expulsion of a wide range of chemically unrelated compounds. It had been proposed that increased extrusion of antimicrobial from biofilm cells might also contribute to resistance. Recent research has addressed the role of the *marRAB* operon and the *acrAB* efflux pump in mediating biofilm resistance (Maira-Litran et al., 2000a; Maira-Litran et al., 2000b). Overall, the results did not support a significant role of *acrAB* and *marRAB* in biofilm resistance to antimicrobial agents. Interestingly, *marRAB* was downregulated in biofilms compared with equivalent stationary phase planktonic cultures (Maira-Litran et al., 2000b). In addition, the deletion of *acrAB* and *marRAB* had no effect on the susceptibility of *E. coli* to ciprofloxacin (Maira-Litran et al., 2000a). Although significant, these studies do not negate the possibility that other efflux pumps may contribute to biofilm resistance.

1.6.2 Role of decreased growth rate in resistance

It is well documented that almost all antimicrobial agents are more effective in killing rapidly growing cells, some even have an absolute requirement for replication (Mah and O' Toole, 2001;Brown et al., 1988). A number of studies have demonstrated that sensitivity to antimicrobial agents increased simultaneously with growth rate in biofilm cells (Evans et al., 1991; Duguid et al., 1992a; Duguid et al., 1992b). However, in many cases the same phenomenon has been observed with planktonic cells (Desai et al., 1998). This indicates that although slow growth undoubtedly contributes to biofilm resistance there are other properties that influence the recalcitrance of biofilms to antimicrobial agents. In addition, it is often very difficult

to differentiate the influence of growth rate from that of cell density, which may also contribute to resistance.

1.6.3 The influence of cell density on resistance

By definition, cell density will be high in a compact, adherent biofilm compared with planktonic culture of equivalent cell number. Therefore, it could be expected that density-dependent events (e.g. quorum sensing) may influence biofilm resistance. However, the precise role of quorum sensing in biofilm resistance is unclear. Most work in this area has focused on *P. aeruginosa* and the production of AHL signalling molecules. Despite an earlier study that showed that mutants deficient in the LasR-LasI quorum sensing system were abnormally sensitive to SDS (Davies et al., 1998), a recent study has shown that similar mutants were unaffected in resistance to antibiotics and detergents (Brooun et al., 2000). The effect of the AI-2 quorum sensing system of *E. coli* on biofilm resistance has yet to be elucidated.

1.6.4 The GSR and biofilm resistance

Recently, it has been proposed that that slow growth exhibited by some cells within a biofilm may not be due to nutrient limitation *per se*, but a result of the *rpoS*-regulated general stress response (Brown and Barker, 1999). The idea that the GSR may play a role in biofilm resistance is an attractive possibility as the GSR is known to lead to cells that are not only more resistant to various environmental stresses but also more virulent (Hengge-Aronis, 2000). Little work has examined the role of the GSR in biofilm resistance to antimicrobial agents. However, studies have indicated that the absence of *rpoS* results in thinner, less complex biofilms in *E. coli* (Adams J L and McLean R J C, 1999). It has been suggested that the high cell density typical of

biofilms may lead to an earlier and more complete general stress response (Brown and Barker, 1999). However, to date, there is no proven link between high cell density and *rpoS* in *E. coli*. In addition, it is unlikely that the GSR can explain the intrinsically high resistance of biofilms to antimicrobial agents as the GSR also occurs in planktonic cells.

1.6.5 Initiation of a biofilm phenotype

The final possibility exists that biofilms exhibit a distinct phenotype with different regulation of resistance-associated genes compared with planktonic culture. The emerging idea is that a biofilm-specific phenotype is induced in a subpopulation of the community that results in the expression of active mechanisms to combat the effects of antimicrobial agents (Mah and O' Toole, 2001; Costerton et al., 1999). Microarray analysis has demonstrated that upon attachment to a surface 38 % of all genes in *E. coli* show altered expression compared with planktonic culture (Prigent-Combaret et al., 1999). Despite microarray analysis, few genes directly related to resistance have been shown to be differentially expressed in biofilms. Recently, it has been demonstrated that biofilm cells exhibit decreased permeability compared with planktonic cells because of an altered porin profile (Prigent-Combaret et al., 1999). A number of other studies support the suggestion that altered porin expression affects the intrinsic resistance of bacteria to antimicrobial agents (Jaffe, 1982). In *Staphylococcus aureus* at least 5 genes are differentially regulated in a biofilm compared with planktonic culture including ClpC ATPase which plays an important role in general stress resistance (Becker et al., 2001).

Although the idea that genes may be differentially regulated in a biofilm is intriguing, it should be emphasised that the culture conditions for biofilm and planktonic cells will be critical if meaningful data are to be gained. Recent studies have neglected to use chemically defined medium and standardised conditions. In many circumstances, gene expression has been compared in planktonic and biofilm cultures that have been subjected to completely different nutrient limitation and that have been grown with vastly different growth rates (Prigent-Combaret et al., 1999; Becker et al., 2001). In order for useful comparisons to be made between planktonic and biofilm cells, the culture conditions must be as similar as possible that is, using chemically defined medium, growing at similar rates, in the same growth phase and under the same nutrient limitation. Only then will it be possible to compare 'like with like' and determine any differences in gene expression between planktonic and biofilm culture.

1.7 AIMS OF THE STUDY

The aim of this study was to:

Determine the influence of nutrient limitation, antimicrobial agents and cell density on the expression of *acrAB*, *marRAB* and *rpoS* for biofilm and planktonic cells and assess the relative importance of these stress responses.

Establish the effect of nutrient limitation, *rpoS*, density-dependent events and growth as a biofilm on physiological changes associated with stationary phase.

Assess the relative contributions of *rpoS*, nutrient limitation, density-dependent events and growth as a biofilm on susceptibility to stress.

2 MATERIALS AND METHODS

2.1 BACTERIAL STRAINS AND GROWTH CONDITIONS

The bacterial strains used in this study are listed in figure 2-1. Strains were stored at –80 °C in Luria Bertani (LB) broth (Oxoid) with 50 % glycerol. At weekly intervals strains were streaked onto LB agar (Oxoid) with the appropriate antibiotic, checked for purity and stored at 4 °C.

Figure 2-1. Bacterial strains and their relevant genotype used in this study

SPECIES, STRAIN	RELEVANT GENOTYPE	REFERENCE, SOURCE
E. coli MC4100	F-Δ(*arg-lac*)*U169 araD139 rpsL 150 ptsF25 flbB5301 rpsR deoC relA1*	(Silhavy, 1984). Hengge-Aronis, Berlin Germany.
E. coli RO200	MC4100 (λRZ5: *rpoS742::lacZ*)	(Lange and Hengge-Aronis, 1994). Hengge-Aronis, Berlin Germany.
E. coli RO91	MC4100 [λRZ5: *rpoS742::lacZ* (hybr)]	(Lange and Hengge-Aronis, 1994). Hengge-Aronis, Berlin Germany.
E. coli RH90	MC4100 *rpoS359::Tn10*	(Lange and Hengge-Aronis, 1991). Hengge-Aronis, Berlin Germany.
E. coli AF633	MC4100 λ.ϕ (P_{uspB}-lacZ)	(Farewell et al., 1998). Farewell, Lund Sweden.
E. coli W4680	(pNN602 *acrAB::lacZ*)	(Ma et al., 1995). Ma, Berkeley USA.
E. coli B306	MC4100 (ϕ*marR25-lacZ*)	(Gambino et al., 1993). Gambino, Ann Arbor USA.
E. coli CF1693	MG1655 (*ΔrelA251::kan ΔspoT207::cml*)	(Xiao et al., 1991). Cashel, Bethesda USA.
E. coli AWS91	R091 (*ΔrelA251::kan*)	This study
Vibrio harveyi BB120	wild type	(Bassler et al., 1994). Bassler, Princeton USA.
Vibrio harveyi BB170	(LuxN::Tn5, sensor 1^-, sensor 2^+)	(Surette and Bassler, 1999). Bassler, Princeton USA.

2.2 CHEMICALLY DEFINED MEDIUM 10 (CDM_{10})

Complete CDM comprised: $MgSO_4$, 0.3 mM; NaCl, 0.05 mM; $(NH)_2HPO_4$, 1.51 mM; KCl, 0.34 mM; $NH_4H_2PO_4$, 1.49 mM; $(NH_4)_2SO_4$, 21 mM; MOPS (3-(*N*-morpholino) propane sulphonic acid, 100 mM (pH 7.2); $FeSO_4$, 1.79 μM; glycerol 50 mM. For nitrogen limited CDM, $(NH)_2HPO_4$ and $NH_4H_2PO_4$ were replaced with 3 mM Na_2HPO_4 and Na_2SO_4 was used as a sulphate source instead of $(NH_4)_2SO_4$. In complete CDM, all nutrients are present in excess such that the medium would support a theoretical extrapolated growth to an optical density (OD 600nm) of 10 in planktonic culture. For nutrient-limited conditions the concentration of one nutrient was reduced so that the population entered stationary phase solely due to the exhaustion of that nutrient. CDM was prepared with population limiting concentrations of carbon, magnesium, nitrogen and phosphate. Iron-restriction was imposed on cells by omitting $FeSO_4$ from the medium.

2.3 INOCULATION AND GROWTH OF PLANKTONIC CULTURE

Cultures were grown at 37 °C with aeration (200 rpm) in baffled, 250 ml conical flasks. Overnight starter cultures were grown in 25 ml volumes and all other cultures were grown in 50 ml volumes. A standard inoculation procedure was followed, overnight cultures were diluted in a ratio 1:100 into fresh CDM. Cultures were always inoculated from overnight cultures that had been grown in CDM with the equivalent nutrient limitation. For carbon limitation glycerol concentrations ranged between 0.05 and 35 mM. For magnesium limitation $MgSO_4$ concentrations ranged between 0.324 μM and 0.0405 mM. For nitrogen limitation $(NH_4)_2SO_4$ concentrations ranged between 0.04 and 40 mM. For phosphate limitation $(NH)_2HPO_4$ concentrations ranged between 0.015 and 0.4 mM. Calibration curves

were constructed by plotting the final stationary phase optical density against the nutrient concentration. The linear portion of the graph indicated the concentrations over which the nutrient limited population density. Above the linear range the population density was limited by factors other than nutrient limitation such as oxygen restriction.

2.4 INOCULATION AND GROWTH OF BIOFILM CULTURES

Biofilms were grown using a modified version of the reproducible nutrient-depleted biofilm developed by (Bühler et al., 1998). Essentially, bacteria were incubated on a filter support placed on a known volume of solidified CDM. Hydrophobic edge membranes (pore size 0.2 μm, 45 mm diameter, Sartorius) were placed in a filter holder (Nalgene) and washed with 5 ml of CDM without the corresponding limiting nutrient. Membranes were then placed on the surface of a CDM agar plate and prewarmed at 37 °C for 1 h. Cells from a nutrient-limited stationary phase culture were diluted to 2 x 10^7cfu / ml in CDM without the limiting nutrient. An aliquot (50 μl) of the bacterial suspension was dropped onto the center of the membrane and the agar plate / membrane was incubated at 37 °C. Bacteria from the biofilm were harvested by scraping the membrane with a sterile glass spreader, resuspending in 5ml of CDM without the appropriate limiting nutrient and vortexing to disperse aggregates. CDM agar was made by adding 1.2 % purified agar (w/v) to CDM. Aliquots (20 ml) of CDM agar were dispensed into sterile petri dishes using a 10 ml automatic pipette (Eppendorf) with disposable tips.

For carbon limitation glycerol concentrations ranged between 1.5 and 15 mM. For nitrogen limitation $(NH_4)_2SO_4$ concentrations ranged between 0.3 and 3 mM. For

phosphate limitation $(NH)_2HPO_4$ concentrations ranged between 0.015 and 0.25 mM. For magnesium limitation $MgSO_4$ concentrations ranged between 2.84 μM and 0.028 mM, but it was found that during prolonged periods of incubation magnesium in the agar was utilised by the bacteria and the population was no longer magnesium limited and growth ceased due to other, unknown factors. Calibration curves were plotted as described above.

2.5 COLONY COUNTS

Colony counts were made using both the spread plate and the Miles-Misra drop plate methods (Miles et al., 1938). For the spread plate method the cell suspension was diluted and known volumes (generally 100 μl) spread on the surface of overdried nutrient agar plates using a sterile glass spreader. For the Miles-Misra method agar plates were divided into an appropriate number of sectors and 20 μl of diluted suspension was dropped onto the surface from a height of approximately 2 cm. The excess liquid was allowed to soak into the agar before the plates were inverted and transferred to a 37 °C incubator. Viable organisms produced colonies on the surface of the agar over the course of incubation at 37 °C. It was assumed that one viable organism produced one colony and the total number of colonies from a given volume multiplied by the dilution factor gave the number of viable organisms in the original suspension. Dilutions were made in 1.5 ml microcentrifuge tubes (Eppendorf). Ten fold dilutions were made in 0.9 ml of either M9 salts medium (Sigma), saline or CDM (without the relevant limiting nutrient) until the calculated number of cells for plating was between 20 and 200 cells for the spread plate method and less than 30 cells for the drop plate method. The reproducibility of these counting procedures was established: 5 series of dilutions with 5 replicate plates for each were subjected to

analysis of variance. The resulting colony counts for the spread plate method are shown in figure 2-2 and the analysis of variance is shown in figure 2-3. The colony counts for the Miles-Misra method are shown in figure 2-4 and the analysis of variance is shown in figure 2-5.

Figure 2-2. Colony counts per plate for five replicate counts as determined using the spread plate method of viable counting.

Replicate count	Replicate plate				
	1	2	3	4	5
1	94	86	98	103	101
2	101	103	84	96	100
3	106	88	73	96	87
4	98	105	102	85	103
5	92	95	94	101	99
Total (T)	491	477	451	481	490
Mean (x)	98.2	95.4	90.2	96.2	98

Figure 2-3. Analysis of variance of five replicate counts as determined using the spread plate method of viable counting.

Source of variance	Sum of squares	Degrees of freedom	Mean squares	Variance ratio
Between counts	210	m-1 4	52.6	0.796
Within counts	1321.6	n.m-m 20	66.08	

Where: n = number of observations per count = 5
m = number of counts = 5
n.m = total number of observations = 25

The tabulated values of F at 4 /20 degrees of freedom at 5 % and 1 % levels are 2.87 and 4.43, respectively. Therefore, the variation between counts is not significantly greater than the variation within counts.

Figure 2-4. Colony counts per plate for five replicate counts as determined using the Miles-Misra method of viable counting.

Replicate count	Replicate plate				
	1	2	3	4	5
1	18	24	24	19	22
2	24	18	17	27	18
3	24	24	23	18	25
4	19	20	19	23	24
5	20	26	20	25	19
Total (T)	105	112	103	112	108
Mean (x)	21	22.4	20.6	22.4	21.6

Figure 2-5. Analysis of variance of five replicate counts as determined using the Miles-Misra method of viable counting.

Source of variance	Sum of squares	Degrees of freedom	Mean squares	Variance ratio
Between counts	13.2	m-1 4	3.3	0.322
Within counts	204.8	n.m-m 20	10.24	

Where: n = number of observations per count = 5
m = number of counts = 5
n.m = total number of observations = 25

The tabulated values of F at 4 /20 degrees of freedom at 5 % and 1 % levels are 2.87 and 4.43, respectively. Therefore, the variation between counts is not significantly greater than the variation within counts.

2.6 SPECTROPHOTOMETRIC MEASUREMENTS OF BACTERIAL GROWTH

The growth of bacterial suspensions was monitored at 600nm. Optical density (OD) measurements were made in disposable plastic cuvettes with a 1 cm light path. As deviation from the Beer-Lambert law occurs when optical density exceeds 0.3 (figure 2-6), samples were diluted sufficiently so that this value was not reached. The relationship between optical density and colony count was determined by inoculating 50 ml of LB medium in a 250 ml conical flask with 100 µl of an overnight *E. coli* MC4100 culture. OD (600 nm) readings were taken throughout the growth curve and at the same time 100 µl volumes were spread in triplicate onto the surface of overdried nutrient agar plates after appropriate 10-fold dilutions had been made in M9. Counts were made following incubation at 37 °C for 24 h and the results were expressed as colony forming units per ml (cfu / ml) and are shown in figure 2-7.

Figure 2-6. Observed versus calculated optical density (600 nm) of suspensions of *E. coli* MC4100 in CDM showing deviation from the Beer-Lambert law for the opacity of bacterial cell suspensions.

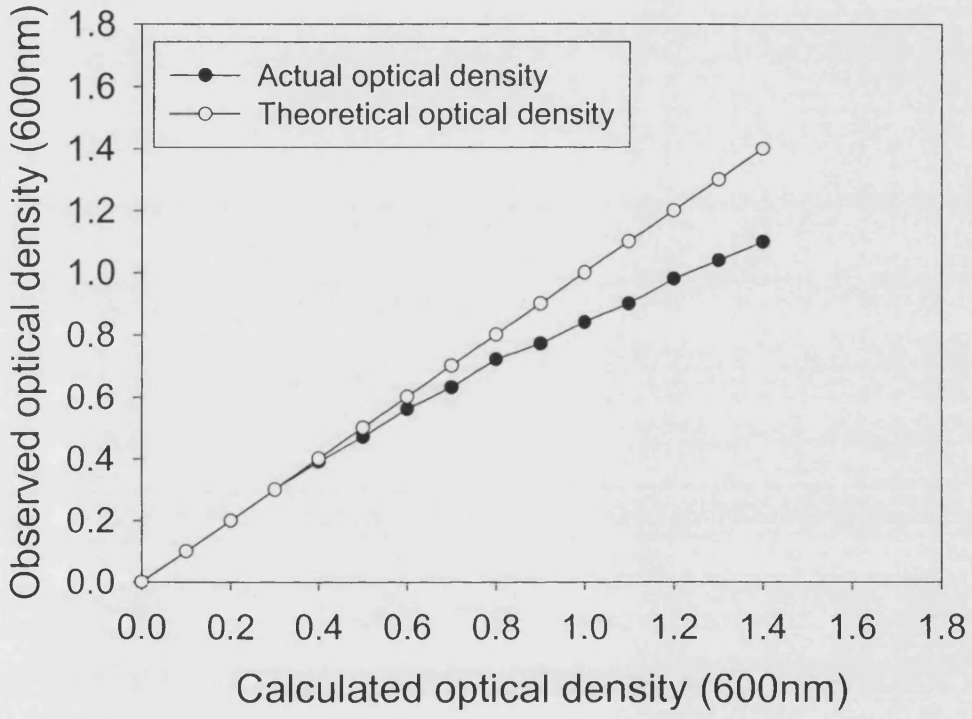
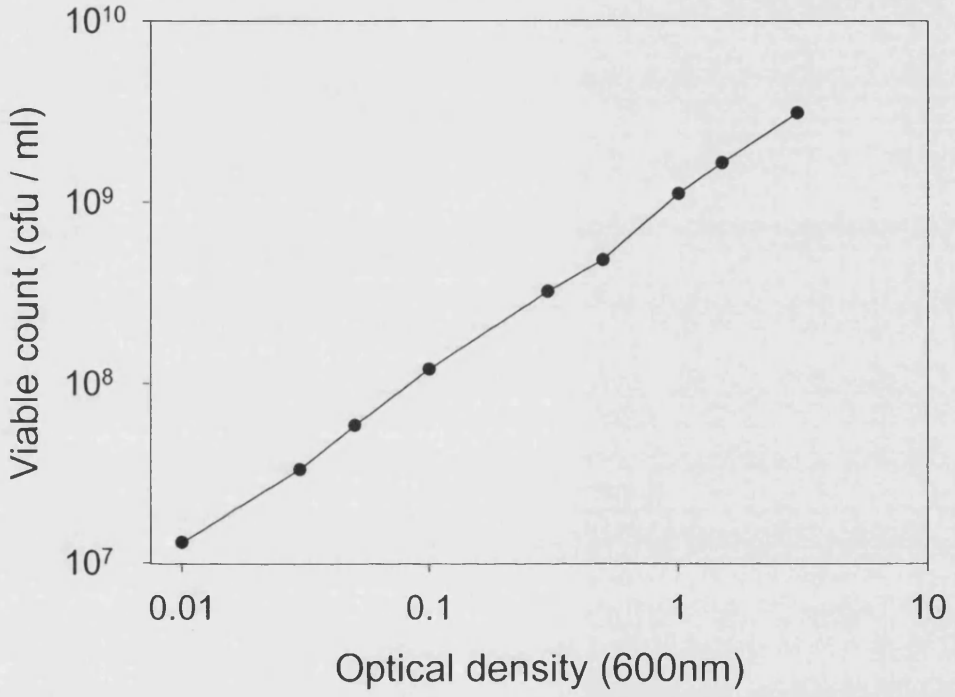


Figure 2-7. The relationship between optical density (600 nm) and viable count for *E. coli* MC4100 grown in Luria-Bertani broth at 37 ° C with aeration.



2.7 P1VIR TRANSDUCTION

2.7.1 Preparation of a high titre P1vir stock

P1vir transduction was carried out essentially as described by (Miller, 1972). A high titre lysate was made by inoculating 50 µl of an overnight culture (18 h after inoculation) of *E. coli* CF1693 into 10 ml of fresh LB broth with 5 mM $CaCl_2$. The culture was grown to an OD (600 nm) of 0.1-0.2 at 37 ° C with aeration then 200 µl was spread onto a fresh LB agar plate to create a lawn of bacteria. The plate was allowed to dry and triplicate aliquots of 5 µl of P1vir phage stock were dropped onto the plate. After overnight incubation at 37 °C plaques created by P1vir lysis were clearly visible on the plate. A sterile pipette tip was used to remove a small plug of agar from the centre of a plaque. The plaque was resuspended in 1 ml of LB with 5 mM $CaCl_2$ and 100 µl of chloroform was added. The phage titre was determined by making serial dilutions of the phage stock in LB with 5 mM $CaCl_2$ and mixing 100 µl of the diluted phage with an equal volume of an exponential culture (OD 600 nm of 0.1-0.2) of *E. coli* CF1693. The phage and bacteria were incubated at 37 °C for 20 min to enable the phage to adsorb and then spread on a fresh LB agar plate. After overnight incubation the phage titre could be determined by counting the number of plaques on the surface of the agar and multiplying this number by the dilution factor to give the number of plaque forming units per given volume.

2.7.2 Preparation of P1vir lysate

Fresh LB broth (5 ml) with 5 mM $CaCl_2$ was inoculated with 5 µl of an overnight culture of CF1693 and the culture grown until an OD of 0.1-0.2. P1vir was pre-adsorbed by adding 10^7 phage to 1 ml of the culture and incubating at 37 °C for 20

min. R-top agar (2.5ml) (Miller, 1972) was added and immediately plated onto a fresh LB plate. After overnight incubation at 37 °C the soft agar was scrapped into a centrifuge tube and the surface of the plate washed with 1 ml of LB and the wash added to the centrifuge tube. Chloroform was added (100 µl) and the tube vortexed for 30 seconds. After 10 min at room temperature the cell debris was pelleted by centrifugation at 8, 000 g for 10 min, 4 °C. The supernatant containing the P1vir lysate was stored at 4 °C.

2.7.3 P1vir transduction

High titre P1vir lysate (approximately 10^{10} pfu/ml) was used to transduce marked alleles from a donor strain to a recipient strain. The recipient strain (R091) was grown up from a single colony inocula to stationary phase at 37 ° C with aeration. An aliquot (1ml) of an overnight culture of the recipient strain was pelleted by centrifugation at 5, 000 g for 10 min and resuspended in 1ml of 0.1 M $MgSO_4$, 5 mM $CaCl_2$. Recipient cells and phage were added to five sterile 1.5 ml microcentrifuge tubes (Eppendorf) in the following ratios:

Tube number	Volume of recipient cells	Volume of P1vir lysate
1	100 µl	0 µl
2	100 µl	1 µl
3	100 µl	10 µl
4	100 µl	50 µl
5	100 µl	100 µl
6	0 µl	100 µl

Tubes 1 and 5 act as controls for reversion and bacterial contamination of the P1vir lysate, respectively. The tubes were vortexed and incubated in a static 37 °C incubator for 30 min to allow phage adsorption. To prevent excessive killing of the recipient cells sodium citrate was then added (100 µl, 1 M) which chelates Ca^{2+} and Mg^{2+} thus preventing readsorption of the phage. LB broth was then added (1 ml) and the tubes were then incubated at 37 °C for 1 h to enable phenotypic expression of the transducer marker. The entire contents of the tubes were spread onto fresh LB agar with sodium citrate (1 M) and the appropriate selective agent and incubated at 30 °C for 48 h. Transductants were tested for the presence of a stable lysogen by serial subculture onto LB with the appropriate selection. Permanent stocks of the resulting transductants were then made.

2.8 β-GALACTOSIDASE ASSAY

β-galactosidase activity was determined essentially as described by (Miller, 1972). Samples equivalent to 1.5 x 10^8 cells were collected and stored at –20 °C before use. Samples were thawed at room temperature and the supernatant discarded after centrifugation at 12,000 g for 3 min. Pellets were resuspended in 0.9 ml of Z-buffer (Miller, 1972) and the chloroform-SDS method of cell permeabilization was used (10 µl of 0.1% SDS and 20 µl chloroform). Samples were then preincubated at 28 °C in a water bath before the addition of 200 µl of the chromogenic substrate *o*-nitrophenyl-β-d-galactopyranoside (4 mg/ml in 0.1 M potassium phosphate, pH 7) (Sigma). The reaction was allowed to continue for 15- 40 min until a mid-yellow colour developed. Sodium carbonate (0.5 ml of 1 M solution) was added to stop the reaction and the samples were centrifuged at 13, 000 g for 3 min. The supernatant was assayed at OD

(470 nm) using Z-buffer as a blank. β-galactosidase activity is reported in Miller units (Miller, 1972). Miller units were calculated as 1000 x OD_{420} / Time at 28 °C x 0.15.

2.9 SDS-PAGE AND IMMUNOBLOT ANALYSIS

Samples corresponding to 3 x 10^9 cells were resuspended in 100 µl of sterile water and an aliquot was used to determine total cellular protein (Lowry, 1951). Samples were boiled with 40 µl SDS-PAGE sample buffer (sodium dodecylsulphate, 2 % w/v; mercaptoethanol, 5% w/v; glycerol, 10% w/v; bromophenol blue, 0.125% w/v in 0.5M Tris buffer). Samples equivalent to 50 µg total cellular protein were separated on 10 % SDS-polyacrylamide gels (Laemmli, 1970) and directly electroblotted onto nitrocellulose membranes (BDH). SDS-PAGE and electroblotting were performed using the respective modules of the Bio-Rad Mini-PROTEAN III system. Blots were blocked overnight in TBS (20 mM Tris-HCl. pH 5.5 and 0.9% NaCl) with 5% milk powder, probed with polyclonal antisera against RpoS (a gift from A. Tanaka, Tokyo), washed with TBS, and incubated with protein-A (Sigma) or goat anti-rabbit IgG alkaline phosphatase conjugate (Dako). The blots were developed either with ECL (Amersham) as a chemiluminescent substrate or with a chromogenic substrate (4-chloro-1-naphthol solution).

2.10 ENZYMATIC ASSAY FOR TREHALOSE ACCUMULATION

Strains MC4100 and RH90 were grown in CDM with nutrient limitation. Stationary-phase cells (6 h after entry) were assayed for trehalose accumulation using a method adapted from a yeast trehalose assay (Parrou and François, 1997). Essentially, trehalose was assayed by measuring glucose production after the breakdown of

trehalose to glucose by the addition of trehalase. Cells (5 x 10^9 cfu) were harvested by centrifugation at 5, 000 g and the pellet resuspended in 0.25 ml of sodium carbonate (0.25 M). After a 4 h incubation at 95 °C the pH was adjusted to 5.2 by the addition of 0.15 ml of acetic acid (1 M) and 0.6 ml of sodium acetate (0.2 M, pH 5.2). Each sample was divided into two aliquots. Porcine trehalase (0.05 U) (Sigma) was added to one aliquot and it was then incubated at 37 °C for 18 h. The other aliquot of each sample acted as a control and was incubated for 18 h at 37 ° C without trehalase. Samples were then centrifuged at 5, 000 g for 3 min and glucose determined using a commercial glucose assay kit (Sigma). Glucose measurements for each sample were normalised for endogenous glucose by subtracting the glucose content of the control aliquot (incubated without trehalase) from the test aliquot (incubated with trehalase).

2.11 FATTY ACID ANALYSIS

Phenotypic differences were assessed by fatty acid methyl ester (FAME) analysis. Cells (50 mg) were harvested into glass test tubes and the FAMEs extracted as described previously (Thompson et al., 1993). Essentially, the bacterial cells were saponified in sodium hydroxide in aqueous methanol (3.75 M NaOH in 50 % methanol), methylated in hydrochloric acid (3 M) in aqueous methanol and the FAMEs were extracted with a hexane / methyl-tert butyl ether solvent mix (1:1). FAME profiles were obtained using a Hewlett-Packard 5890 series II gas chromatograph with a 25 m (length) x 0.2 mm (internal diameter) x 0.33 μm (film thickness) phenyl methyl silicone capillary column and a flame ionization detector (FID). The analysis was performed under the control of the Microbial Identification System software (Microbial ID Inc., Newark, DE, USA). Samples (1 μl) were injected at 250°C and the column temperature was steadily increased from 170 °C to

270 °C at a rate of 5 °C per min. Peaks were identified by their retention time on the column and the proportion of each in the profile was calculated from the relative peak areas. The mean FAME profile from the replicates was used to construct a library. A dendrogram based on the Euclidean distance (Equation 1) between pairs of strains and clustered using the unweighted pair group method with arithmetic averages (UPGMA) was produced. Significant differences in the proportions of individual fatty acids were determined by analysis of variance (Fry, 1993).

Equation 1

$$d_{ab} = \frac{\sqrt{(X_{iA} - X_{iB})^2 + (X_{2A} - X_{2B})^2 \cdots\cdots (X_{tA} - X_{tB})^2}}{\sqrt{t}},$$

Where d_{ab} is the average Euclidean distance, X_{iA} and X_{iB} are the states of character i (i=1, 2, ..., t) for two individuals and t is the total number of characters.

2.12 ASSAY FOR THE PRODUCTION OF LUXS HOMOLOGUE QUORUM SENSING MOLECULES

2.12.1 Preparation of cell free culture fluids

E. coli strains RH90 and MC4100 were grown in CDM with nutrient limitation. Populations were limited at 'high' (approximately OD 600 nm 2.00) and 'low' (approximately OD 600 nm 0.05) densities with carbon, magnesium, nitrogen and phosphate limitation. Samples (1 ml) were taken at various time points throughout the growth cycle. Cell-free culture fluids were prepared by centrifuging samples at 13, 000 g for 3 min in a microcentrifuge at 4 °C. The supernatant was passed through a 0.2 μm filter (Whatman, 13 mm GD/X disposable) and stored at –20 °C.

2.12.2 Determination of light-production throughout the growth cycle for *V. harveyi* BB170

Cell-free culture fluids were tested for the presence of signalling molecules that could induce luminescence in the *Vibrio harveyi* reporter strain BB170 according to the method of Surette and Bassler (Surette and Bassler, 1998). The *V. harveyi* reporter was grown for 16 h at 30 °C with aeration (150 rpm) in Autoinducer Bioassay (AB) medium (Greenberg et al., 1979). AB medium contained 0.3M NaCl, 50 mM, 0.102 M $MgSO_4$, 0.2 % casaminoacids, 10 mM K_2HPO_4 (pH 7.0), 1mM L-arginine and 1% glycerol. The overnight culture was diluted 1:5,000 into fresh AB medium. Luminescence was measured as a function of cell density by measuring light production at various times throughout the growth cycle. Light production was measured by removing triplicate, 30 µl aliquots from the culture and assaying them in a black microtitre plate using a TopCount (Packard instruments) set in luminescence mode. The cell density was measured by removing aliquots at the same time that samples were taken for light production measurement, diluting the samples in saline and spreading the dilutions onto solid L-marine medium (LM medium, 0.7 M NaCl, 1% Bacto tryptone, 0.5% Bacto yeast extract and 1.5 % agar). Plates were inverted and incubated at 30 °C overnight, the resulting colonies counted the next day and relative light units (RLU) calculated. RLU are (counts min^{-1} ml^{-1} x 10^3) / colony forming units ml^{-1})

2.12.3 Assay for production of signalling molecules

The quorum sensing activity of *E. coli* RH90 and MC4100 was assayed after growth under nutrient limitation. Cells were grown and cell-free culture fluids harvested as previously described. Aliquots (500 µl) of cell free culture fluid were added to 4.5 ml

of fresh AB medium in 27 ml Sterilin tubes (Sterilin) and the mixture inoculated with a 1 µl of an overnight culture of *V. harveyi* BB170. A positive control consisted of 4.5 ml of fresh AB with 500 µl of cell-free culture fluid from an overnight culture of BB170, inoculated as described above. Fresh AB with 10 % CDM and inoculated with BB170 served as a negative control to ensure that any increase in luminescence was due to signaling substances present in the cell-free culture fluid of *E. coli* and not a growth medium component. An additional sample of AB without any additional supernatant inoculated with BB170 was also used. All samples were incubated at 30 °C, 150 rpm for approximately 3.5 h (that is, until the culture without supernatant had reached mid-log and luminescence was at a minimum) and then triplicate 30 µl aliquots were taken from each culture and the light production assayed using a TopCount. Luminescence of samples is expressed as percentage luminescence of the positive control.

2.13 'RING OF FIRE' ASSAY FOR GENE EXPRESSION

A simple, rapid method for detecting gene expression in LacZ reporter strains based on the method of Gilbert (P. Gilbert, personal communication 2000) was used to determine the effect of exposure to household and food products on the expression of *acrAB*, *marRAB* and *rpoS*. Essentially, LB agar was overlaid with 200 µl X-gal (20 mg/ml 5-Bromo-4-chloro-3-indolyl beta-D-galactopyranoside in dimethylformamide, Sigma) and the plate left at 37 °C for 5 h to allow the evaporation of dimethylformamide. The surface of the agar was inoculated with 100 µl of an LB grown overnight culture of *E. coli* strains R091, W4680 or B306 that had been diluted to 10^7 cfu / ml. Wells (6 mm diameter) were cut in the center of the agar and 50 µl of a household / food product was placed in the well. Aqueous products

were solidified with 0.05% agar. Plates were incubated at 37 °C for 16 h and then transferred to 4 °C to ensure full colour development. Gene expression from the reporter fusion was deemed to have occurred if blue colonies were present.

2.14 ANTIMICROBIAL SUSCEPTIBILITY TESTING

2.14.1 Determination of the minimum inhibitory concentration (MIC) of antimicrobial agents

The susceptibility of *E. coli* to a number of antimicrobial agents was determined. Aliquots (10 ml) of Isosensitest broth (Oxoid) containing serial two-fold dilution of each antimicrobial agent were inoculated with a total of 1 x 10^7 organisms. Growth was assessed visually after 48 h of incubation at 37 °C. The MIC was defined as the lowest concentration of antimicrobial agent that inhibited visual growth

2.14.2 Determination of the kinetics of kill of antimicrobial agents

The kinetics of kill of a number of antimicrobial agents were determined for stationary phase (6 h after entry) nutrient-limited *E. coli* MC4100 and RH90 and LB grown W4680 and B306. The kinetics of kill was determined in either CDM (without the appropriate limiting nutrient) for cells grown with nutrient limitation or in M9 for cells grown in LB broth. The appropriate concentration of each antimicrobial agent was added to a 10 ml of either CDM or M9. A control comprised of CDM or M9 without the antimicrobial agent. Tubes containing the antimicrobial agent and control tubes were inoculated with a total of 1 x 10^7 cells. Aliquots (100 µl) were removed at the beginning of the experiment and every 10 min subsequently for 50 min. Viable counts were determined by the Miles-Misra method. Dilutions for viable counts were performed using inactivator broth (Oxoid).

2.15 ASSAY TO DETERMINE THE SUSCEPTIBILITY OF *E. COLI* TO HEAT

The susceptibility of nutrient-limited (6 h after entry into stationary phase) *E. coli* MC4100 and RH90 to heat at 52 °C was determined. Aliquots (10 ml) of CDM (minus the relevant limiting nutrient) were prewarmed to 52 °C. Control tubes comprised of CDM at room temperature. Prewarmed and control tubes were inoculated with a total of 1 x 10^7 cells. Aliquots (100 µl) were removed at the beginning of the experiment and every 10 min subsequently for 50 min. Serial dilutions were performed in CDM and viable counts determined using the Miles-Misra method.

2.16 ASSAY TO DETERMINE THE SUSCEPTIBILITY OF *E. COLI* TO DRYING

2.16.1 Determination of the susceptibility of planktonic *E. coli* to drying

The susceptibility of planktonic stationary phase (6 h after entry) nutrient-limited *E. coli* MC4100 and RH90 to drying was determined. Pieces of filter paper (1 cm^2) (Whatman) were inoculated with 10 µl of culture to give 1 x 10^7 organisms per square of filter paper. Filter paper squares were places in a petri dish and transferred to a 20 °C incubator. Triplicate samples were removed from the incubator every 1 h for a period of 5 h. Individual samples were placed in a microcentrifuge tube (Eppendorf) containing 1 ml of CDM (minus the limiting nutrient) and macerated using sterile scissors. Serial dilutions were then performed in CDM (minus the limiting nutrient) and viable counts determined using the Miles-Misra method. As a control, viable counts were determined for triplicate samples (of each strain, with each nutrient limitation) prior to incubation at 20 °C.

2.16.2 Determination of the susceptibility of biofilm *E. coli* to drying.

The susceptibility of stationary phase biofilm nutrient-limited *E. coli* MC4100 and RH90 to drying was determined. Biofilms were grown as described previously except; the inoculum was divided into four aliquots, which was placed on the membrane equidistant apart so that four separate biofilms grew on each membrane. When the biofilms had reached 6 h into stationary phase the membrane was removed, cut into four sections (each containing a biofilm) and each section was placed in a sterile petri dish. Biofilms were transferred to a 20 °C incubator. Triplicate samples were removed from the incubator at time intervals between 1 and 215 h. Individual samples were placed in a 30 ml disposable tube (Sterilin) with 5 ml of CDM (minus the limiting nutrient) and vortexed to remove the biofilm from the membrane. Serial dilutions were then performed in CDM (minus the limiting nutrient) and colony counts determined using the Miles-Misra method. As a control, colony counts were determined for triplicate samples (of each strain, with each nutrient limitation) prior to incubation at 20 °C

2.17 Data Analysis

On selected data one-way analysis of variance (ANOVA, SPSS 9.0 for Windows) was performed to determine if significant differences existed between mean values. Tukey's Honest Significant Difference (HSD, SPSS 9.0 for Windows) was used to determine which means differed from one another. These differences are indicated on the corresponding figures. Bars that are not labelled with the same letter are significantly different ($P \leq 0.05$).

3 THE EFFECT OF GROWTH CONDITIONS ON *RPOS* EXPRESSION

3.1 INTRODUCTION

The *rpoS* encoded sigma factor (σ^S) is the master regulator of many stationary phase controlled genes (Weichart et al., 1993; Lange and Hengge-Aronis, 1991). The regulation of *rpoS* is complex and cellular levels of σ^S are controlled at the levels of transcription, translation and proteolysis (Hengge-Aronis, 1999; Lange and Hengge-Aronis, 1994; Loewen et al., 1993). The expression of many σ^S-regulated genes is modulated by other global regulators such as H-NS, cAMP-CRP, IHF and Fis (Hengge-Aronis R, 1999). There is also interplay between other regulatory networks, notably the stringent response and the alarmone ppGpp (Gentry et al., 1993). Numerous other small molecules, RNAs and proteins have also been implicated in affecting *rpoS* levels including Crl (Pratt and Silhavy, 1998), inorganic phosphate (Shiba et al., 1997), UDP-glucose (Hengge-Aronis et al., 1995) and the small RNAs, *dsrA* (Sledjeski et al., 1996) and *oxyS* (Altuvia et al., 1997). Cell density has also been included among possible factors affecting *rpoS* levels (Hengge-Aronis, 1999). Much evidence for density-dependent effects has been indirect (Huisman and Kolter, 1994) or has relied on the use of spent culture medium (Sitnikov et al., 1996) but recently its influence on expression has been demonstrated in a chemostat model (Liu et al., 2000). However, to date the individual influences of growth rate, cell density and nutrient limitation on *rpoS* have not been elucidated.

The aim of this study has been to examine the effect of cell density and specific nutrient limitation on *rpoS* expression in both planktonic and biofilm culture. We have used chemically defined medium (CDM_{10}) with carbon, magnesium, nitrogen and phosphate limitation to control population density.

3.2 RESULTS

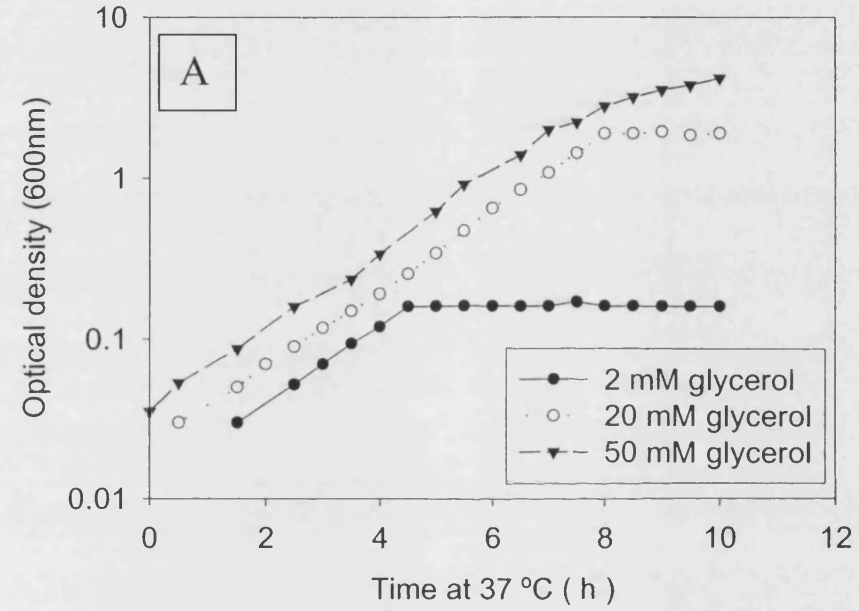
3.2.1 Carbon limitation in planktonic culture

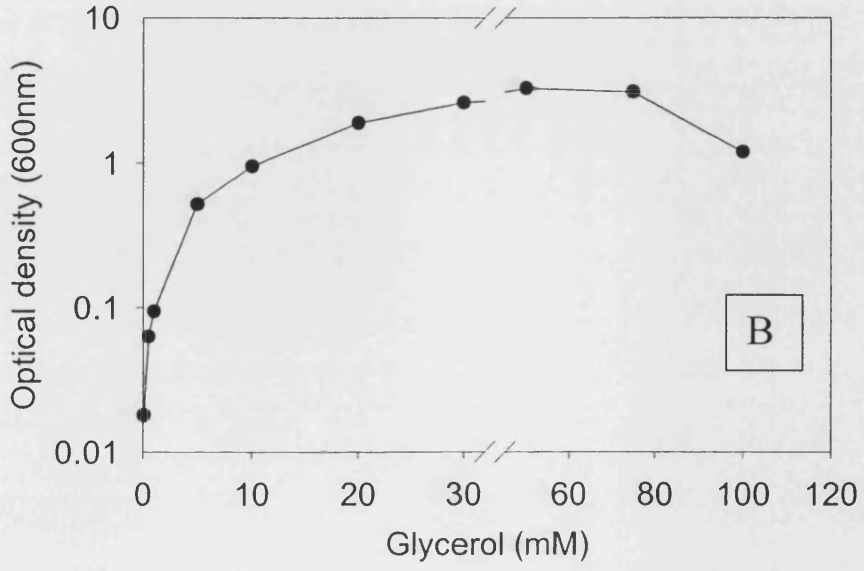
The growth of *E. coli* MC4100 in CDM_{10} containing varying amounts of glycerol was examined. Concentrations of glycerol were (mM) 0.05, 0.10, 0.50, 1.0, 5.0, 10.0, 20.0, 30.0, 50.0, 75.0, 100.0. Several of the resultant growth curves (off-set for clarity) and a plot of maximum (stationary phase) OD (600 nm) against time are illustrated in fig. 3-1. Plotting the maximum OD (600 nm) reached by a culture against the initial glycerol concentration showed that glycerol concentrations below 30 mM affected the density at which stationary phase was entered. At concentrations in excess of 30 mM the population density was limited by factors other than carbon limitation, probably by oxygen depletion. It is interesting to note that at the highest glycerol concentration tested (100 mM) the population density was actually lower than that for 75 mM. Examination of a sample of the 100 mM stationary phase culture microscopically (results not shown) indicated that the cells were exhibiting unusual morphology (long and filamentous cell shape), possibly due to the high osmotic pressure.

Examination of the carbon-limited growth curves (shown with 2 and 20 mM glycerol) showed that the cultures entered stationary phase suddenly, without prolonged slowing of growth rate. The growth curve of a culture grown with excess glucose (50

mM) shows a gradual slowing of growth rate as the cells enter stationary phase.

Figure 3-1. Growth of *E. coli* MC4100 in CDM_{10} with various concentrations of glycerol (A) and calibration curve to show the effect of glycerol concentration on final stationary phase density for *E. coli* MC4100 grown in CDM_{10} at 37 °C with aeration (B).

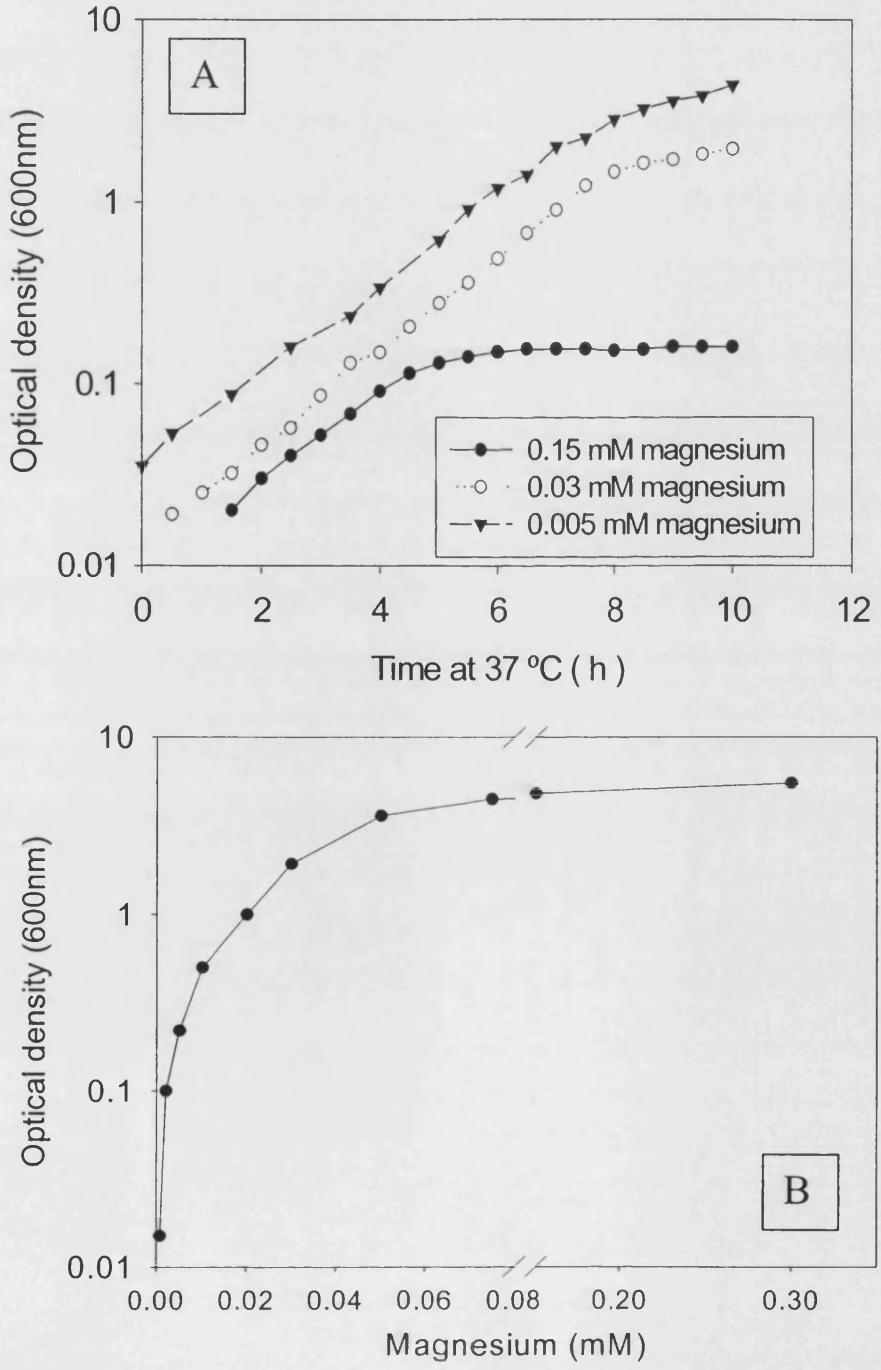




3.2.2 Magnesium limitation in planktonic culture

The growth of *E. coli* MC4100 in CDM_{10} containing varying amounts of magnesium was examined. Concentrations of magnesium were (mM) 0.00075, 0.001, 0.005, 0.01, 0.02, 0.03, 0.05, 0.075, 0.1, 0.3. Several of the resultant growth curves (off-set for clarity) and a plot of maximum (stationary phase) OD (600 nm) against time are illustrated in fig. 3-2. Plotting the maximum OD (600 nm) reached by a culture against the initial magnesium concentration showed that magnesium concentrations below 0.05 mM affected the density at which stationary phase was entered. At concentrations in excess of 0.05 mM the population density was limited by factors other than magnesium limitation. Examination of the growth curves showed that entry into stationary phase because of magnesium limitation (0.005 and 0.03 mM) was more gradual compared with carbon-limited cultures.

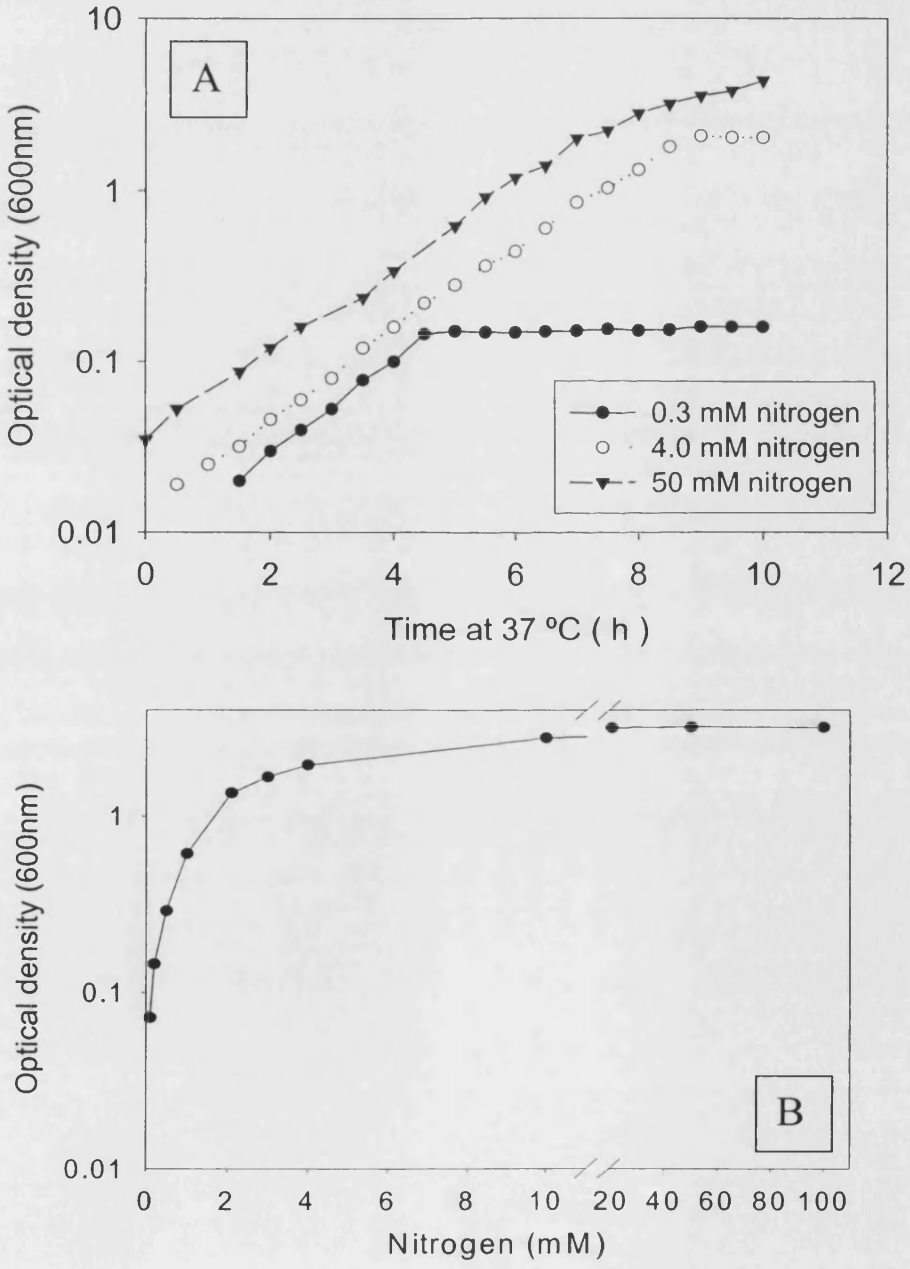
Figure 3-2. Growth of *E. coli* MC4100 in CDM_{10} with various concentrations of magnesium (A) and calibration curve to show the effect of magnesium concentration on final stationary phase density for *E. coli* MC4100 grown in CDM_{10} at 37 °C with aeration (B).



3.2.3 Nitrogen limitation in planktonic culture

The growth of *E. coli* MC4100 in CDM_{10} containing varying amounts of nitrogen was examined. Concentrations of nitrogen were (mM) 0.10, 0.21, 0.5, 1.0, 2.1, 3.0, 4.0, 10.0, 20.0, 50.0, 100.0. Several of the resultant growth curves (off-set for clarity) and a plot of maximum (stationary phase) OD (600 nm) against time are illustrated in fig. 3-3. Plotting the maximum OD (600 nm) reached by a culture against the initial nitrogen concentration showed that nitrogen concentrations below 10.0 mM affected the density at which stationary phase was entered. At concentrations in excess of 10.0 mM the population density was limited by factors other than nitrogen limitation. Examination of the growth curves showed that entry into stationary phase as a result of nitrogen limitation (0.3 and 4.0 mM) occurred suddenly and without a gradual change in growth rate. These growth curves were similar in appearance to those produced by carbon limitation. A growth curve for cells with excess nitrogen is shown (50 mM) and it can be seen that there is a gradual change in growth rate.

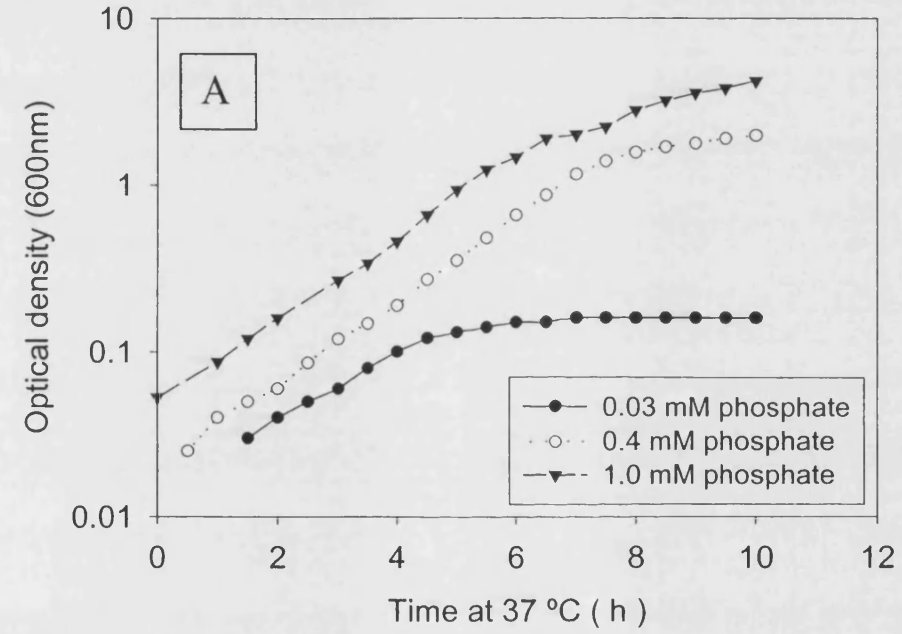
Figure 3-3. Growth of *E. coli* MC4100 in CDM_{10} with various concentrations of nitrogen (A) and calibration curve to show the effect of nitrogen concentration on final stationary phase density for *E. coli* MC4100 grown in CDM_{10} at 37 °C with aeration (B).

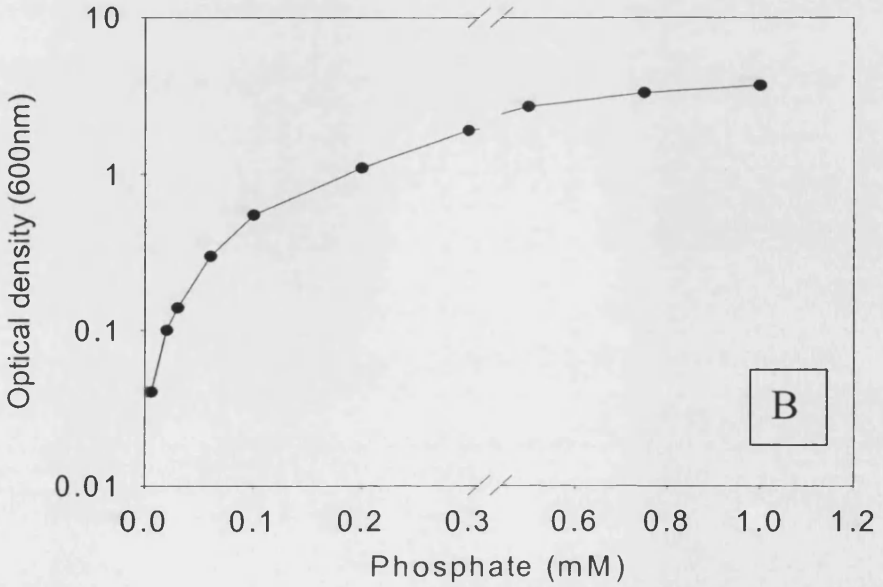


3.2.4 Phosphate limitation in planktonic culture

The growth of *E. coli* MC4100 in CDM_{10} containing varying amounts of phosphate was examined. Concentrations of phosphate were (mM) 0.006, 0.02, 0.03, 0.06, 0.1, 0.2, 0.3, 0.5, 0.75, 1.0. Several of the resultant growth curves (off-set for clarity) and a plot of maximum (stationary phase) OD (600 nm) against time are illustrated in fig. 3-4. Plotting the maximum OD (600 nm) reached by a culture against the initial phosphate concentration showed that phosphate concentrations below 0.5 mM affected the density at which stationary phase was entered. At concentrations below 0.5 mM phosphate, the population density was restricted by phosphate limitation. Examination of the growth curves showed that entry into stationary phase under phosphate limitation (0.03 and 3 mM) occurred gradually, with a slowing of growth rate.

Figure 3-4. Growth of *E. coli* MC4100 in CDM_{10} with various concentrations of phosphate (A) and calibration curve to show the effect of phosphate concentration on final stationary phase density for *E. coli* MC4100 grown in CDM_{10} at 37 °C with aeration (B).

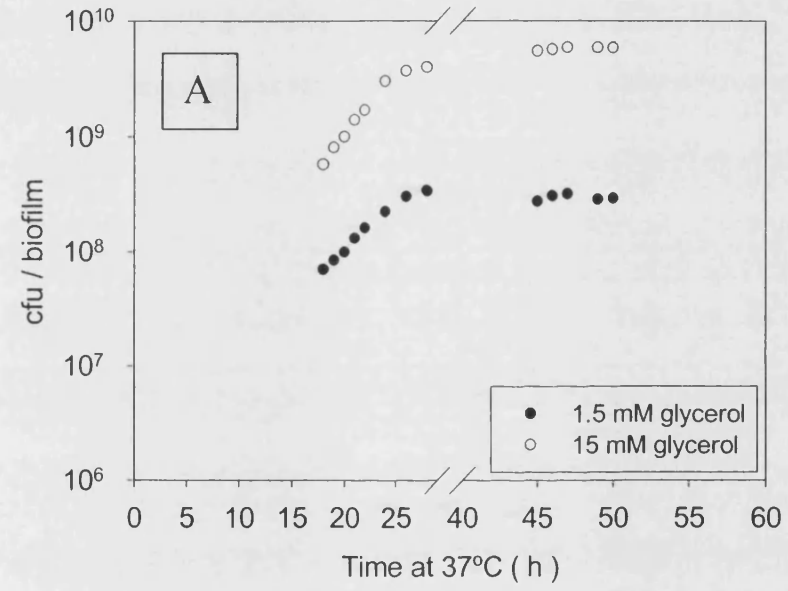


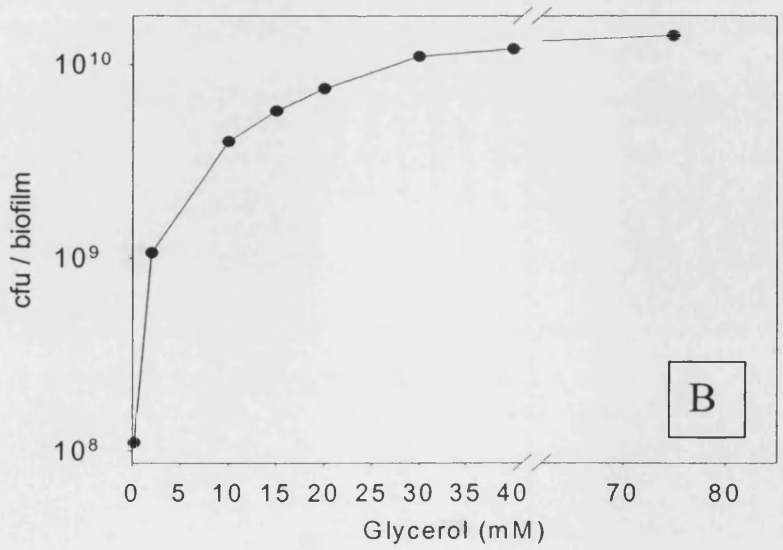


3.2.5 Carbon limitation in biofilm culture

The growth of *E. coli* MC4100 on membranes on CDM_{10} containing varying amounts of glycerol was examined. Concentrations of glycerol were (mM) 0.2, 2.0, 10.0, 15.0, 20.0, 30.0, 40.0, 75.0. Several of the resultant growth curves and a plot of maximum (stationary phase) OD (600 nm) against time are illustrated in fig. 3-5. Plotting the maximum OD (600 nm) reached by a culture against the initial glycerol concentration showed that glycerol concentrations below 30 mM affected the density at which stationary phase was entered. At concentrations greater than 30 mM the population density was limited by factors other than carbon limitation, probably by oxygen depletion.

Figure 3-5. Growth of biofilm *E. coli* MC4100 on CDM_{10} with various concentrations of carbon (A) and calibration curve to show the effect of carbon concentration on final stationary phase yield (colony forming units / biofilm) for *E. coli* MC4100 grown on a membrane on CDM_{10} at 37 °C (B).

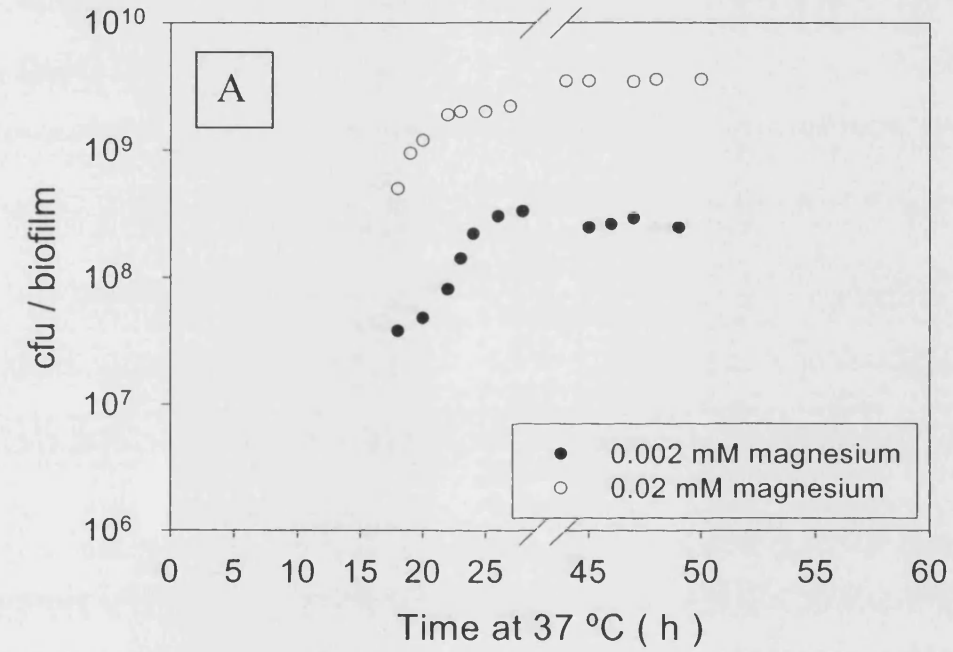


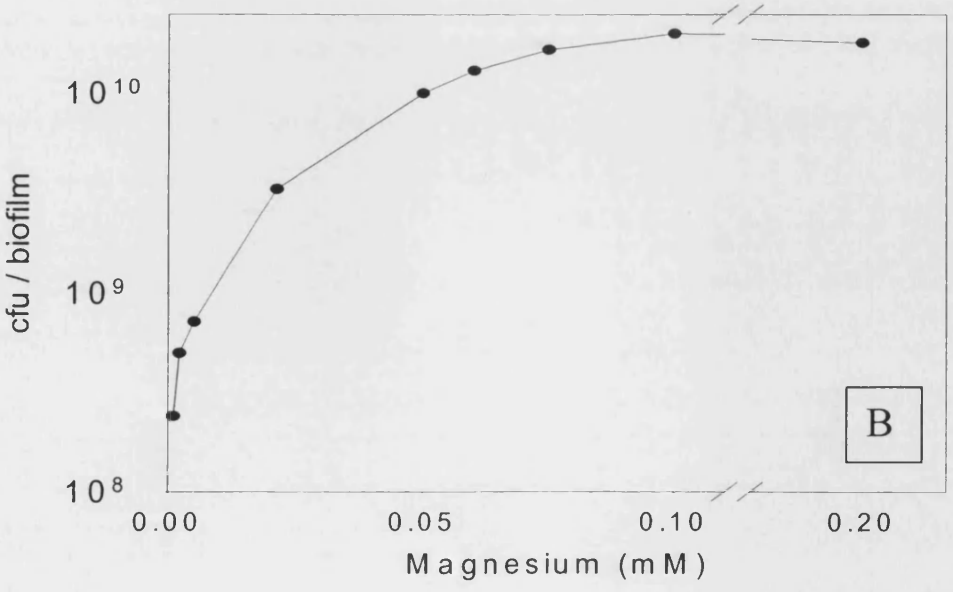


3.2.6 Magnesium limitation in biofilm culture

The growth of *E. coli* MC4100 on membranes on CDM_{10} containing varying amounts of magnesium was examined. Concentrations of magnesium were (mM) 0.001, 0.002, 0.005, 0.02, 0.05, 0.06, 0.075, 0.1, 0.2. Several of the resultant growth curves and a plot of maximum (stationary phase) OD (600 nm) against time are illustrated in fig. 3-6. Plotting the maximum OD (600 nm) reached by a culture against the initial magnesium concentration showed that magnesium concentrations below 0.075 mM affected the density at which stationary phase was entered. At concentrations in excess of 0.075 mM the population density was limited by factors other than magnesium limitation.

Figure 3-6. Growth of biofilm *E. coli* MC4100 on CDM_{10} with various concentrations of magnesium (A) and calibration curve to show the effect of magnesium concentration on final stationary phase yield (colony forming units / biofilm) for *E. coli* MC4100 grown on a membrane on CDM_{10} at 37 °C (B).

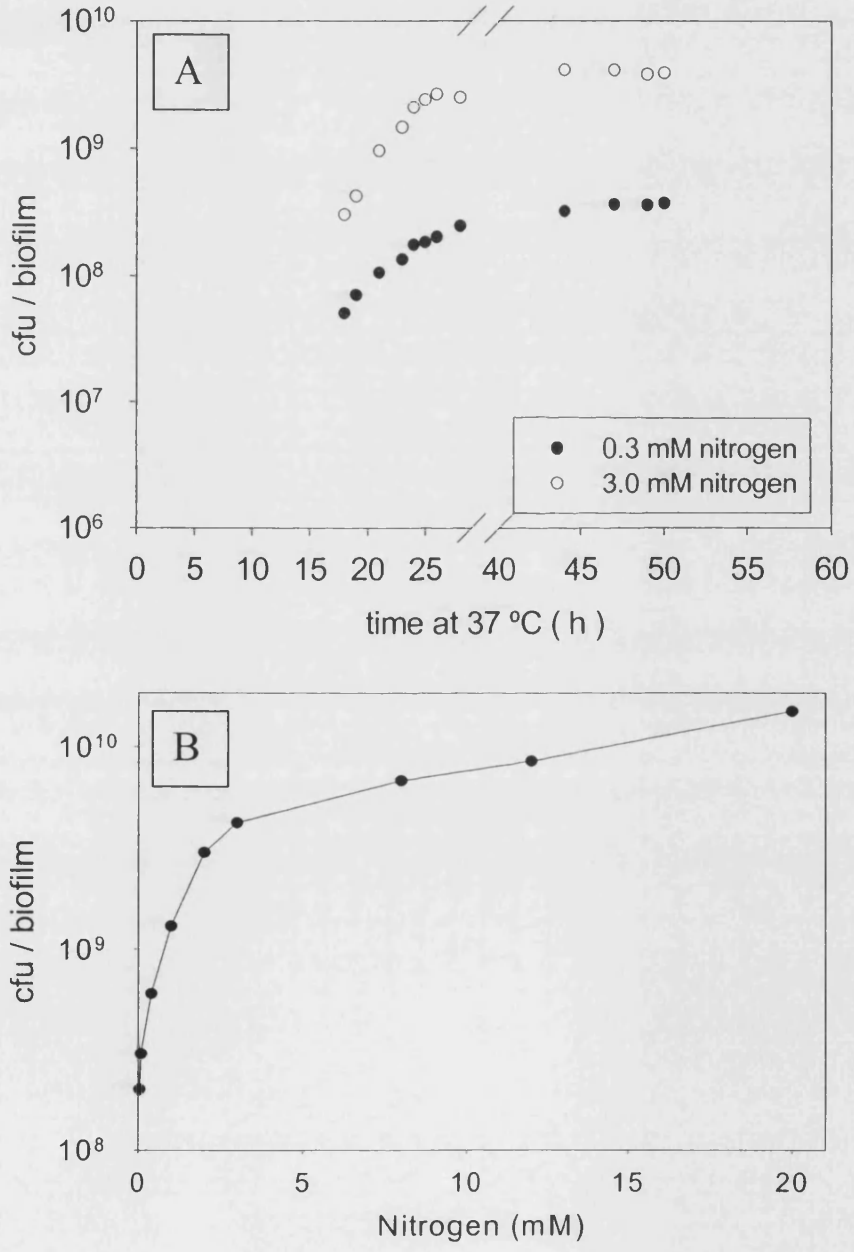




3.2.7 Nitrogen limitation in biofilm culture

The growth of *E. coli* MC4100 on membranes on CDM_{10} containing varying amounts of nitrogen was examined. Concentrations of nitrogen were (mM) 0.04, 0.08, 0.4, 1.0, 2.0, 3.0, 8.0, 12, 20.0. Several of the resultant growth curves and a plot of maximum (stationary phase) OD (600 nm) against time are illustrated in fig. 3-7. Plotting the maximum OD (600 nm) reached by a culture against the initial nitrogen concentration showed that nitrogen concentrations below 3.0 mM affected the density at which stationary phase was entered. At concentrations greater than 3.0 mM the population density was limited by factors other than nitrogen limitation.

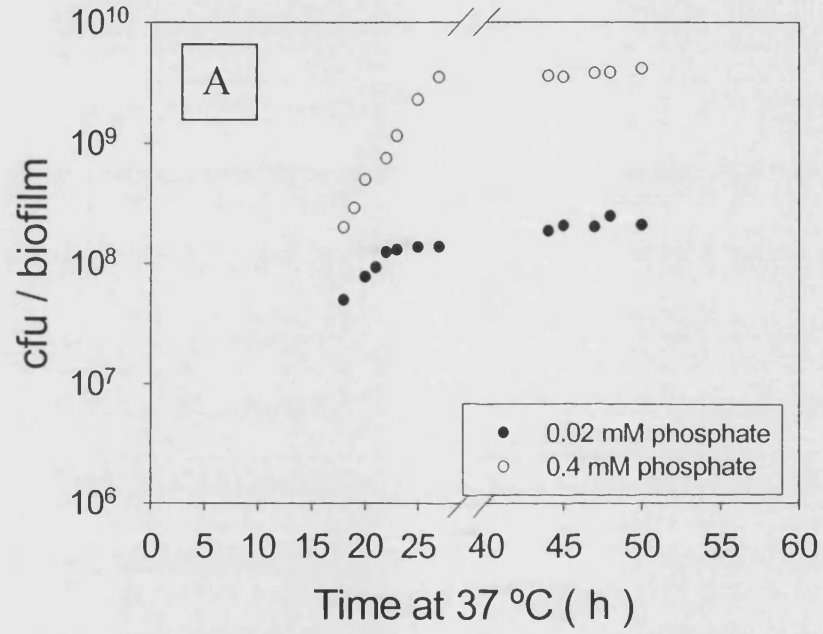
Figure 3-7. Growth of biofilm *E. coli* MC4100 on CDM_{10} with various concentrations of nitrogen (A) and calibration curve to show the effect of nitrogen concentration on final stationary phase yield (colony forming units / biofilm) for *E. coli* MC4100 grown on a membrane on CDM_{10} at 37 °C (B).

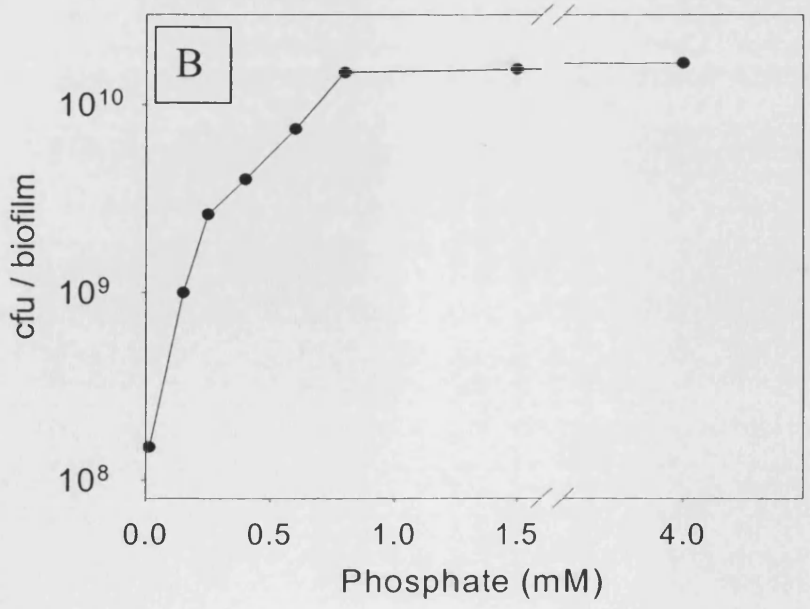


3.2.8 Phosphate limitation in biofilm culture

The growth of *E. coli* MC4100 on membranes on CDM_{10} containing varying amounts of phosphate was examined. Concentrations of phosphate were (mM) 0.015, 0.15, 0.25, 0.4, 0.6, 0.8, 1.5, 4.0. Two of the resultant growth curves and a plot of maximum (stationary phase) OD (600 nm) against time are illustrated in fig. 3-8. Plotting the maximum OD (600 nm) reached by a biofilm against the initial phosphate concentration showed that phosphate concentrations below 0.8 mM affected the time at which stationary phase was entered. At concentrations of 0.8 mM phosphate and greater, the population density was limited by factors other than phosphate limitation.

Figure 3-8. Growth of biofilm *E. coli* MC4100 on CDM_{10} with various concentrations of phosphate (A) and calibration curve to show the effect of phosphate concentration on final stationary phase yield (colony forming units / biofilm) for *E. coli* MC4100 grown on a membrane on CDM_{10} at 37 °C (B).





3.2.9 Transcriptional and post-transcriptional control of *rpoS* in relation to nutrient limitation and cell density as determined by gene fusion and immunoblot analysis

The expression of *rpoS* under conditions of nutrient imitation was examined for both planktonic and biofilm cultures using ß-galactosidase gene fusions. A late-translational fusion was used to examine the post-transcriptional control of *rpoS*. The late-translational fusion (strain RO91) encodes a hybrid protein carrying 247 of a total of 329 amino acids of σ^S (Lange and Hengge-Aronis, 1994). The fusion is inserted 742 nucleotides into the *rpoS* coding region. This fusion can also account for protein stability as it contains the turnover element required for proteolysis and is therefore, degraded concomitantly with σ^S (Muffler et al., 1996). The transcriptional fusion (strain RO200) is derived from the late-translational fusion and is identical except for a 90-bp insertion upstream of the eighth codon of *lacZ*. This insertion ensures that this fusion cannot be translated into protein by read through as it provides stop codons in all three reading frames (Lange and Hengge-Aronis, 1994). Both fusions are single copy, stable and chromosomally integrated.

The expression of these *rpoS*-fusions was examined in planktonic cultures grown in CDM_{10} with limiting concentrations of carbon, magnesium, nitrogen and phosphate. The transcriptional fusion exhibited no induction during entry into stationary phase (fig. 3-9, 3-10). In contrast, the late-translational fusion showed high level induction under conditions of magnesium and phosphate limitation (12- and 14-fold respectively) (fig. 3-11.). Surprisingly, there was no induction in response to carbon and nitrogen limitation. These results were confirmed by immunoblot analysis (fig. 3-13). It was noted that conditions causing *rpoS* induction (magnesium and phosphate

limitation) resulted when there was a gradual change in growth rate upon entry into stationary phase. Carbon- and nitrogen-limited cells entered stationary phase suddenly. No density-dependent effects were apparent when this was repeated with cells limited to a low population density (stationary phase density OD 600nm 0.05). The transcriptional fusion (fig. 3-9, 3-10) showed no induction under any of the conditions tested and the late-translational fusion showed induction only under conditions of magnesium and phosphate limitation (fig. 3-11, 3-12.).

The induction of *rpoS* in response to nutrient limitation was strongly influenced by the mode of growth as biofilm grown cells exhibited distinctly different expression patterns than planktonic culture. The induction of the late-translational fusion was examined throughout the growth cycle in carbon-, magnesium-, nitrogen- and phosphate-limited biofilms at both high (between 4 and 5.5 x 10^9 cfu / biofilm) and low (between 5 and 7 x 10^8 cfu / biofilm) cell density. In contrast to the planktonic cultures, biofilm cultures showed strong *rpoS* induction (in excess of 8-fold) under all nutrient limitations tested (Fig. 3-14, 3-15.). The highest level of induction was exhibited by phosphate-limited biofilms where expression was 22-fold greater than the basal level. Analogous to the planktonic cultures, there were no density-dependent differences in *rpoS* induction. *RpoS* was strongly expressed at both high and low cell densities. The induction of the transcriptional fusion was not examined under the conditions of biofilm growth.

Figure. 3-9. Expression of *rpoS742::lacZ* transcriptional fusion (open circles) throughout the growth cycle (open triangles) for 'high-density' planktonic *E. coli* RO200 grown in CDM_{10} with carbon (A), magnesium (B), nitrogen (C) and phosphate (D) limitation.

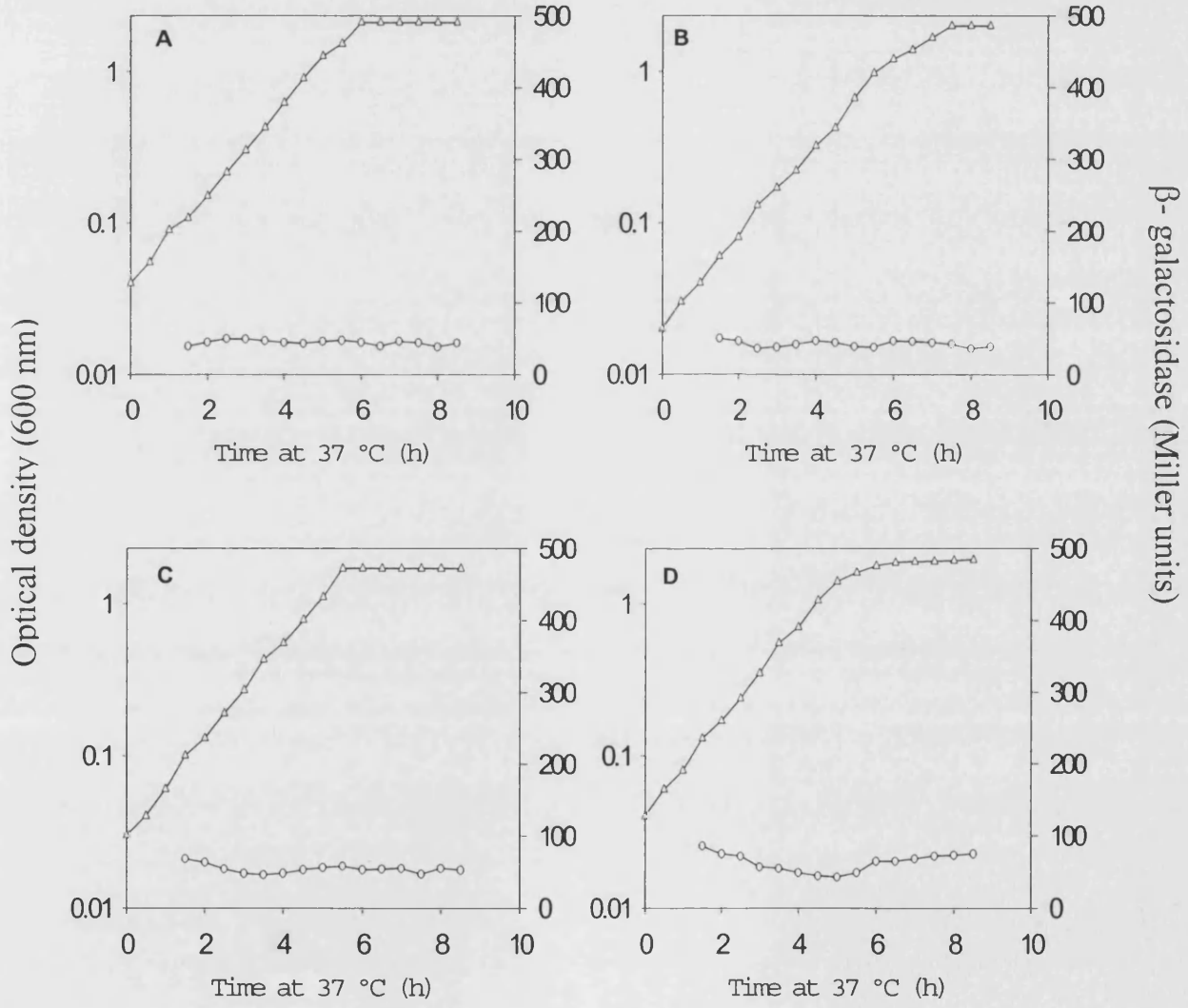


Figure. 3-10. Expression of *rpoS742::lacZ* transcriptional fusion (open circles) throughout the growth cycle (open triangles) for 'low-density' planktonic *E. coli* RO200 grown in CDM_{10} with carbon (A), magnesium (B), nitrogen (C) and phosphate (D) limitation.

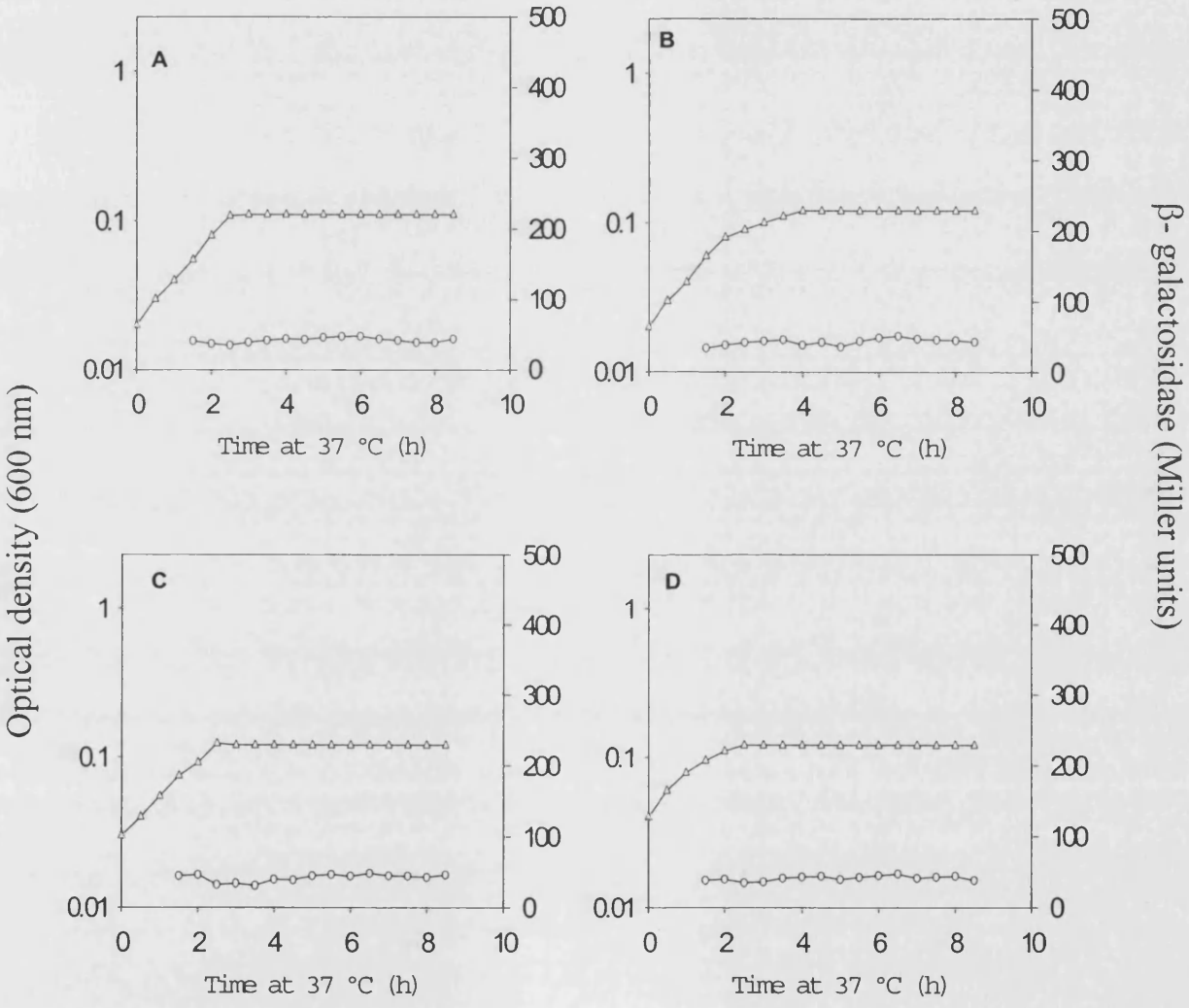


Figure. 3-11. Expression of *rpoS742::lacZ* late-translational fusion (open circles) throughout the growth cycle (open triangles) for 'high-density' planktonic *E. coli* RO91 grown in CDM_{10} with carbon (A), magnesium (B), nitrogen (C) and phosphate (D) limitation.

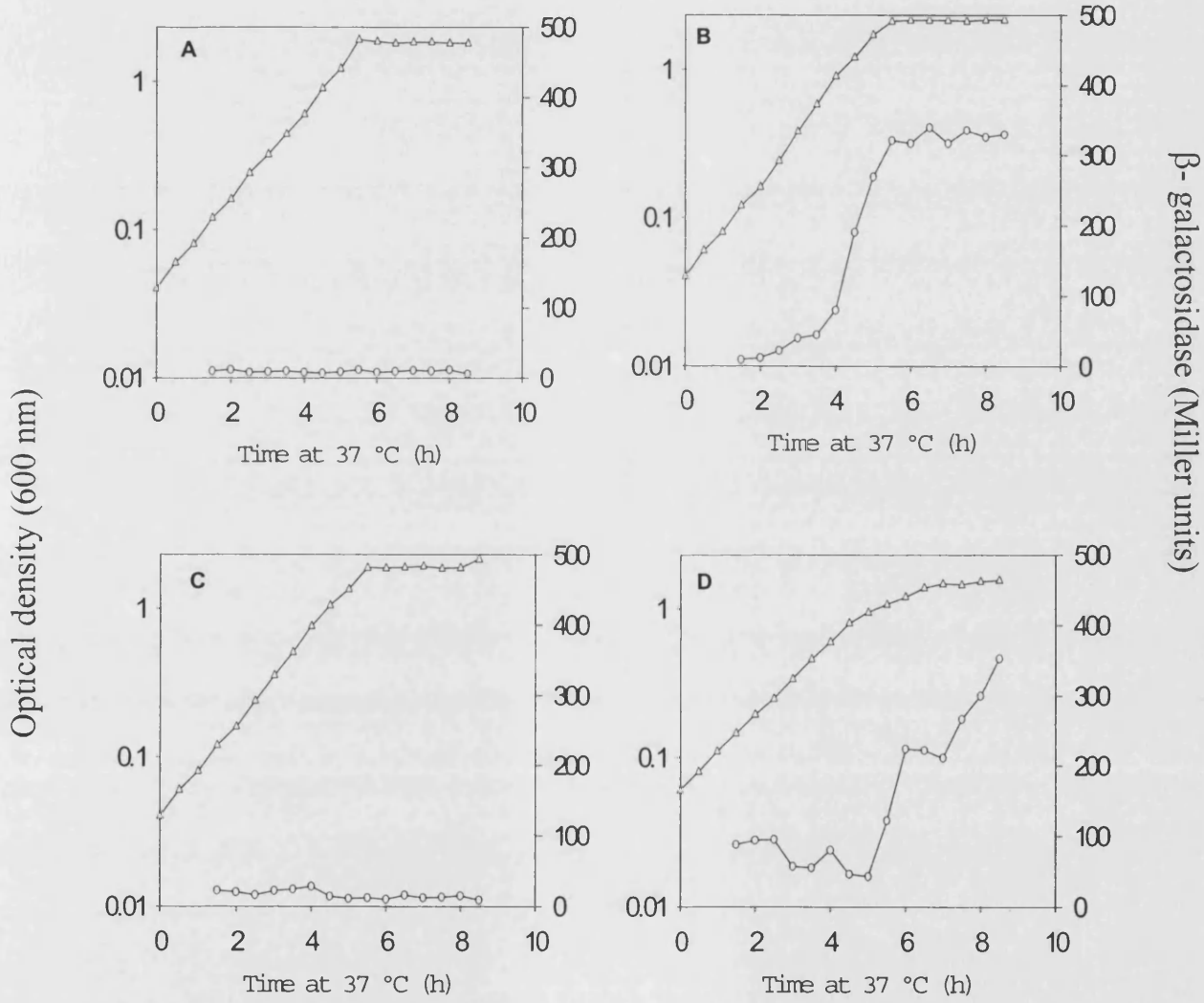


Figure. 3-12. Expression of *rpoS742::lacZ* late-translational fusion (open circles) throughout the growth cycle (open triangles) for 'low-density' planktonic *E. coli* RO91 grown in CDM_{10} with carbon (A), magnesium (B), nitrogen (C) and phosphate (D) limitation.

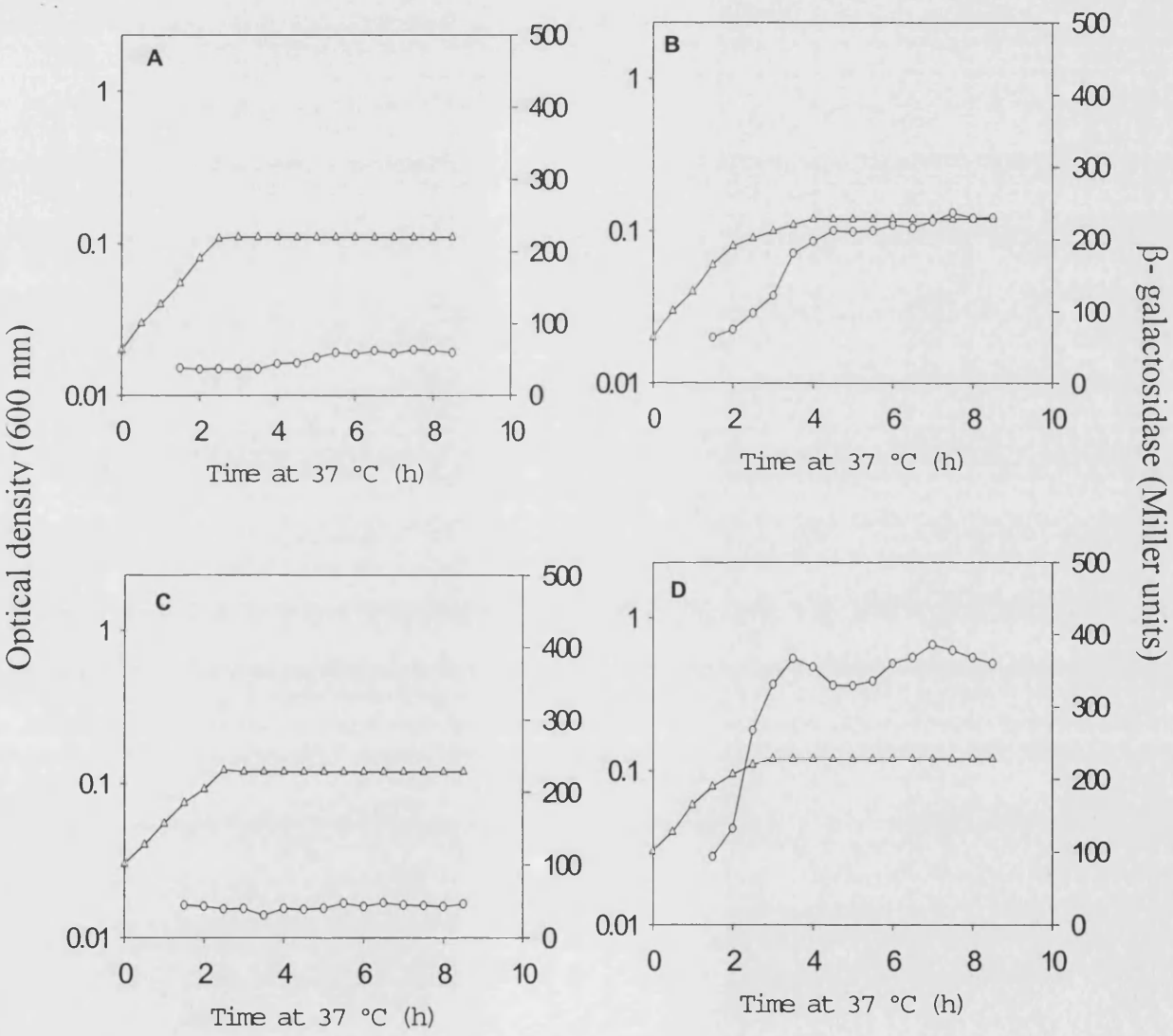


Figure. 3-13. Western blot analysis of *rpoS* expression in response to nutrient limitation for stationary phase (2 hours after entry) high density, planktonic *E. coli* RO91 grown in CDM_{10} with carbon (lane 1), magnesium (lane 2), nitrogen (lane 3) and phosphate (lane 4) limitation. A negative control of *E. coli* RH90 (Δ *rpoS)* is shown in lane 5 and a positive control of *E. coli* MC4100 (grown in LB until 4 hours into stationary phase) is shown in lane 6.

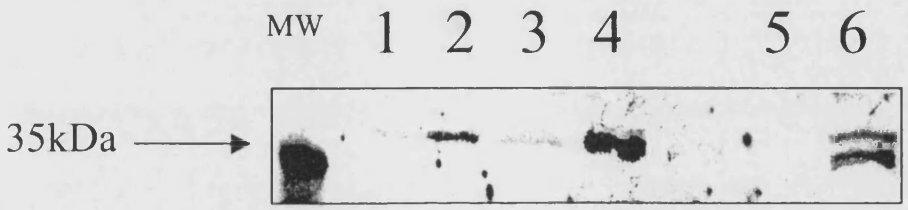


Figure. 3-14. Expression of *rpoS742::lacZ* translational fusion (open circles) throughout the growth cycle (open triangles) for 'high-density' biofilm *E. coli* RO91 grown in CDM_{10} with carbon (A), magnesium (B), nitrogen (C) and phosphate (D) limitation.

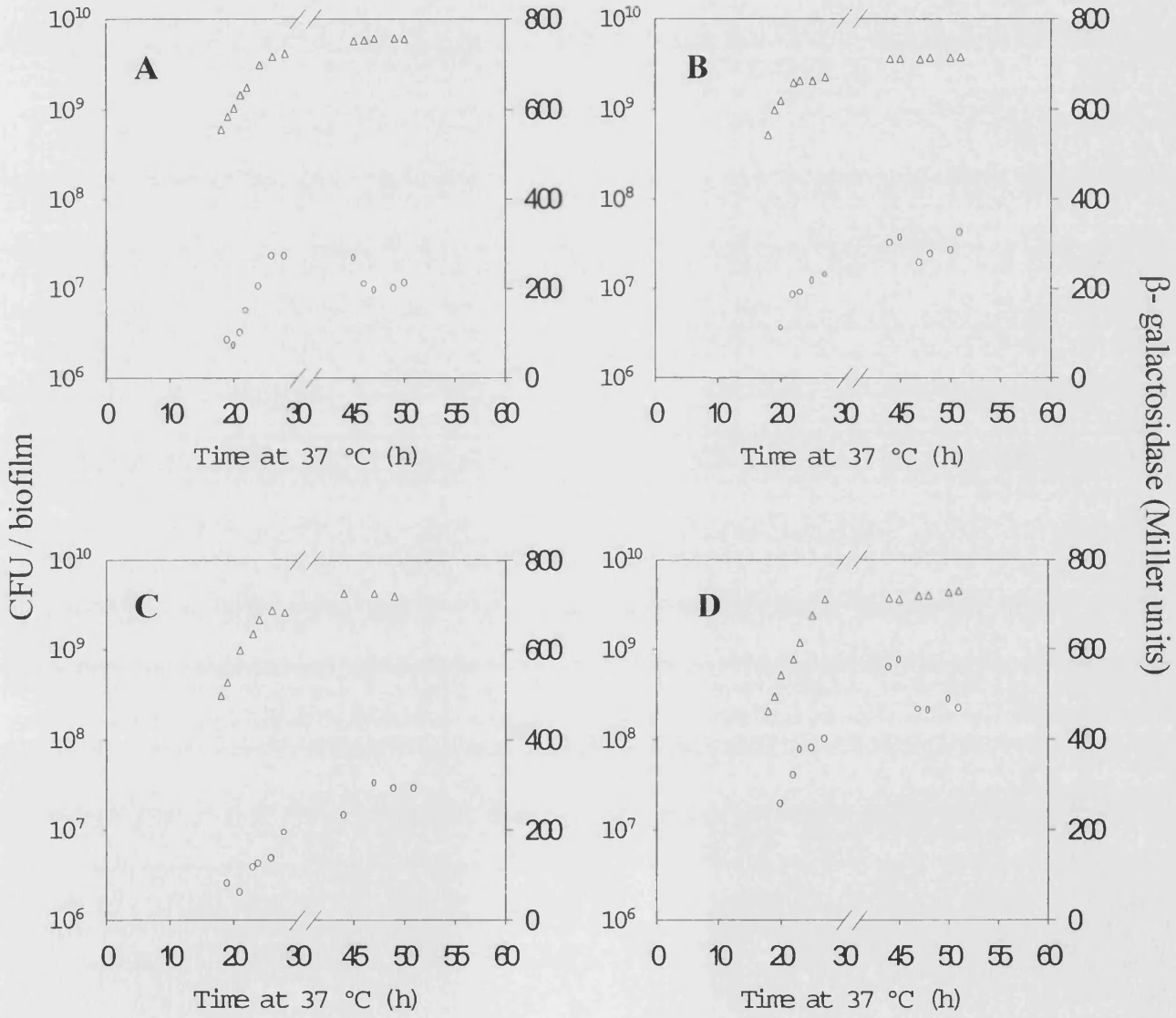
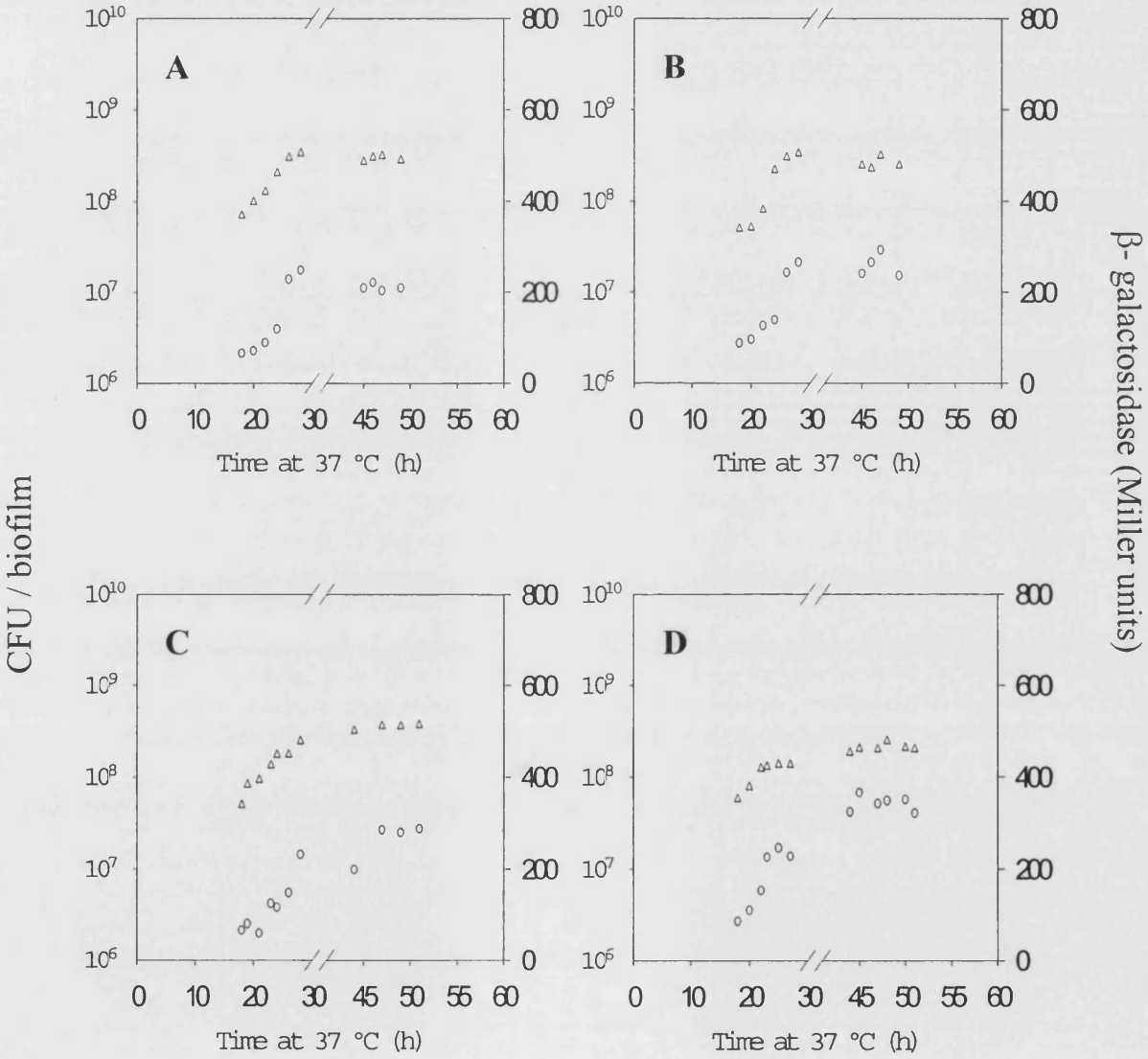


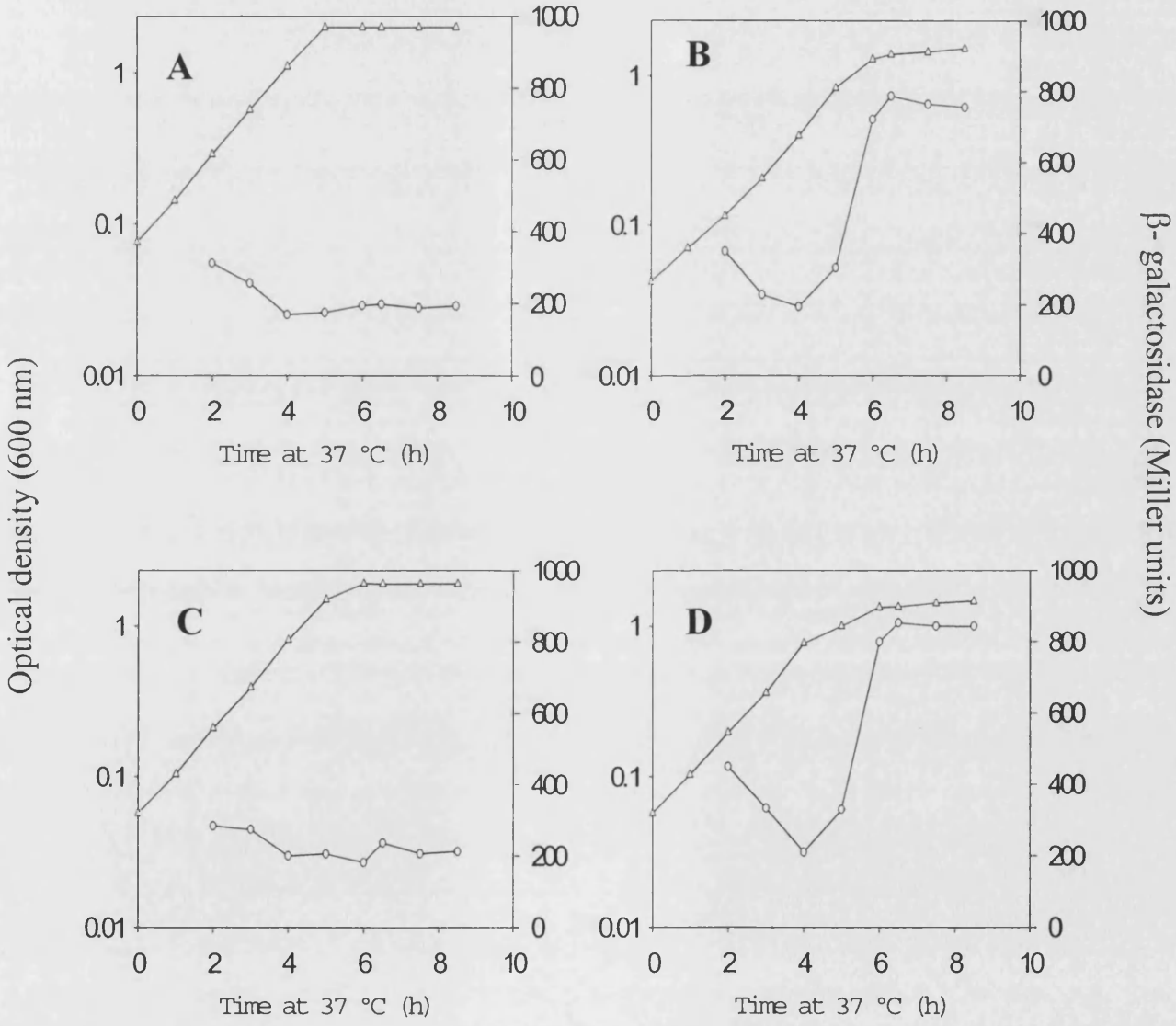
Figure. 3-15. Expression of *rpoS742::lacZ* translational fusion (open circles) throughout the growth cycle (open triangles) for 'low-density' biofilm *E. coli* RO91 grown in CDM_{10} with carbon (A), magnesium(B), nitrogen (C) and phosphate (D) limitation.



3.2.10 Induction of the *rpoS*-regulated *uspB* gene in response to nutrient limitation

The induction of *uspB* in response to nutrient limitation was examined. The *uspB* gene has a single, *rpoS* dependent promoter. A β -galactosidase gene fusion was used to monitor *uspB* induction. Strain AF633 contains the *uspB* promoter (up to the *Msc*I site within the gene) fused to *lacZ* from pTL61T (Farewell A et al., 1998). *UspB* induction was examined in high-density, planktonic nutrient-limited cultures (fig. 3-16.). *UspB* was strongly induced (4-fold) in response to magnesium and phosphate limitation but there was no induction under carbon and nitrogen limitation. These results corroborate those obtained for *rpoS* expression in planktonic culture.

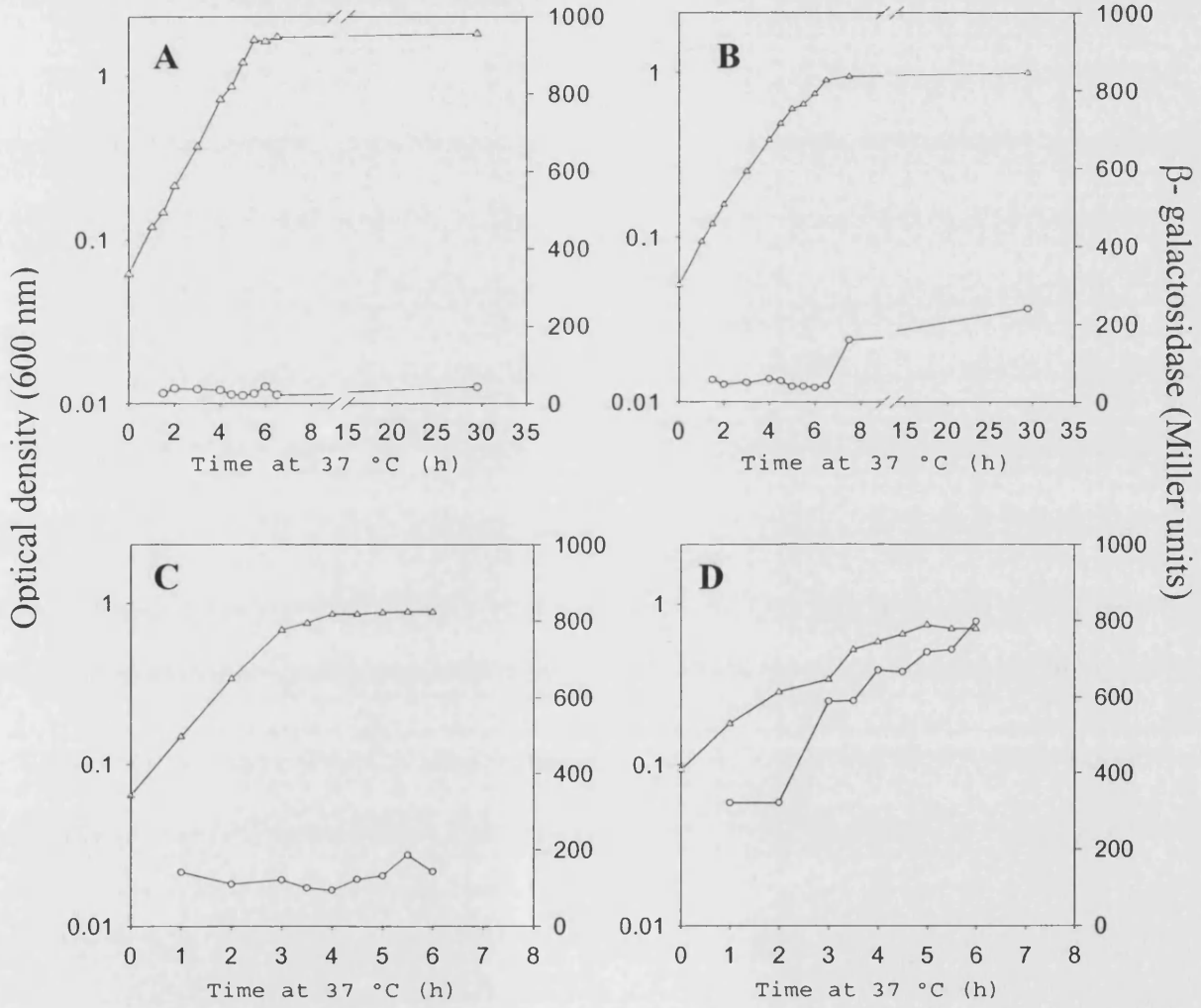
Figure. 3-16. Expression of *uspB::lacZ* (open circles) throughout the growth cycle (open triangles) for 'high -density' planktonic *E. coli* AF633 grown in CDM_{10} with carbon (A), magnesium (B), nitrogen (C) and phosphate (D) limitation.



3.2.11 Effect of iron restriction on *rpoS* induction in planktonic growth

As previous studies have reported that *rpoS* is strongly induced in response to carbon limitation (Schweder et al., 1996; Lange and Hengge-Aronis, 1994), attempts were made to explain why *rpoS* was not induced in planktonic culture under the carbon limitation conditions used in this study. In previous studies, carbon limitation has been achieved using modified M9 medium (Miller, 1972) with glucose as the carbon source. It is proposed that *rpoS* is induced if cells are grown in M9 with no additional iron and that the studies listed above may have examined *rpoS* induction in response to the combined stress of carbon and iron restriction. To test this hypothesis planktonic cultures were grown in M9 with and without iron (1.7 μM iron sulphate) with 0.1 % glucose. It was found that the omission of iron caused exceptionally high induction of *rpoS* and that this induction was not seen if iron was included in the growth medium (fig. 3-17). It is important to note that the omission of iron had a deleterious affect on growth rate. The basal level of *rpoS* expression was higher (over 100 Miller units) in M9 compared with CDM_{10} (25 Miller units). This was then repeated using carbon-limited CDM_{10} without additional iron. There was an slight increase in *rpoS* expression if iron was omitted from the medium but the level of induction was much less dramatic compared with M9 without iron (fig. 3-17.). In addition, there was only a minimal alteration in growth rate if iron was omitted from CDM_{10}.

Figure. 3-17. Expression of *rpoS742::lacZ* late-translational fusion (open circles) throughout the growth cycle (open triangles) for 'high-density' planktonic *E. coli* RO91 grown in CDM_{10} with carbon limitation (20 mM glycerol) and excess iron (A), no additional iron (B) and in M9 with 0.1 % glucose and excess iron (C) and no additional iron (D).



3.2.12 Influence of growth rate on *rpoS* induction in planktonic growth

It had been noted that *rpoS* expression in planktonic culture seemed to correlate with a change in growth rate. Therefore, it was decided to perturb deliberately the growth rate of cells grown in CDM_{10} with a limiting concentration of carbon in order to ascertain whether this would cause *rpoS* induction. The late-translational *rpoS* reporter fusion (RO91) and the late-transcriptional fusion (RO200) were used to monitor *rpoS* induction in response to a decrease in growth rate. Cells were grown in CDM_{10} with a population-limiting concentration of glycerol (20 mM). Growth rate was perturbed by either temperature shift (37 °C to 30 °C) or by decreased aeration (200 to 0 rpm) prior to entry into stationary phase. *RpoS* was induced 3-fold upon temperature shift (fig. 3-19), concurrent to a decrease in growth rate. In addition *rpoS* was induced in response to decreased aeration, but only if cells were switched at a low population density (OD 600nm of 0.075) (fig. 3-18). At higher cell density (OD 600nm of 0.9), the sudden decrease in aeration did not cause *rpoS* expression, and there was no gradual change in growth rate as the cells suddenly ceased growing. If the cells were switched to low aeration at a low population density an increase in *rpoS* was seen, as was a perturbation of growth rate. The transcriptional fusion showed no induction under any of the conditions tested.

Figure. 3-18. Effect of switch in aeration (from 200 to 0 rpm) on *rpoS742::lacZ* late-translational fusion induction for planktonic *E. coli* RO91 grown in CDM_{10} with limiting carbon and subjected to: no switch in aeration (A), switch in aeration at high cell density (OD 600nm of 0.9) (B) and switch in aeration at low cell density (OD 600nm of 0.075) (C). Switch in aeration is represented by an arrow.

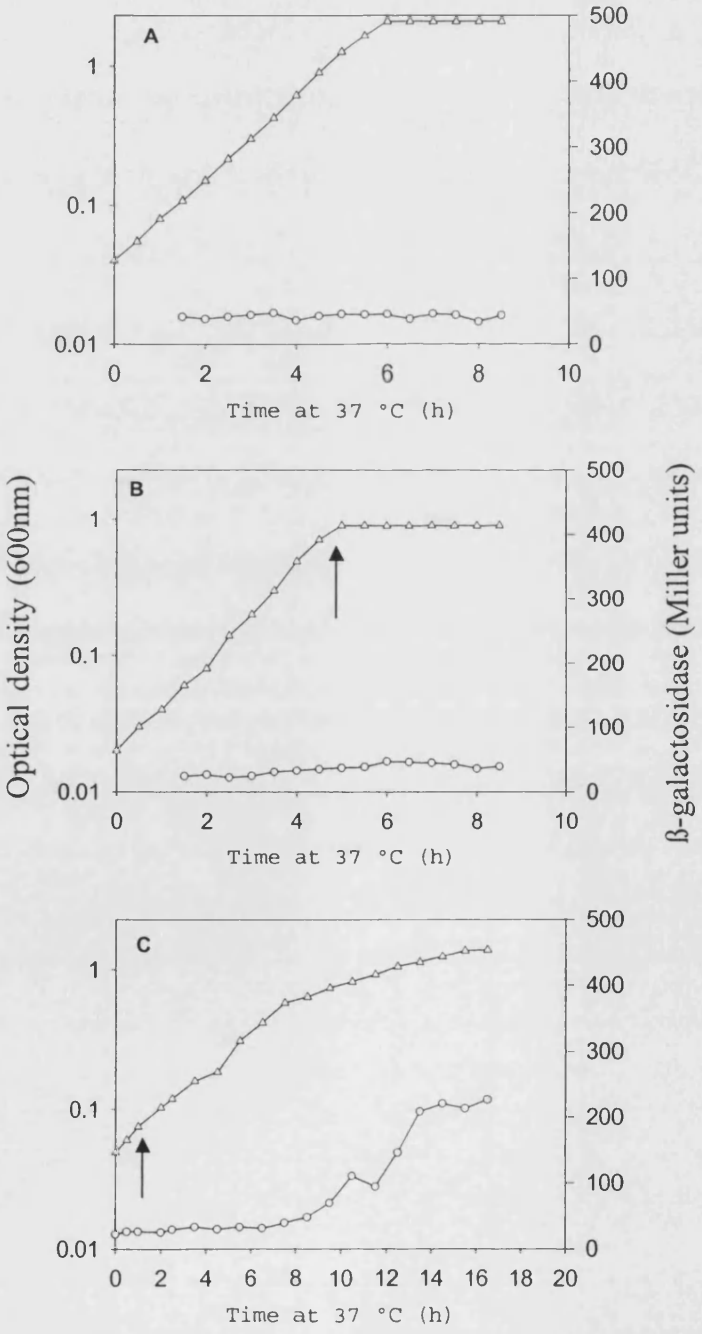
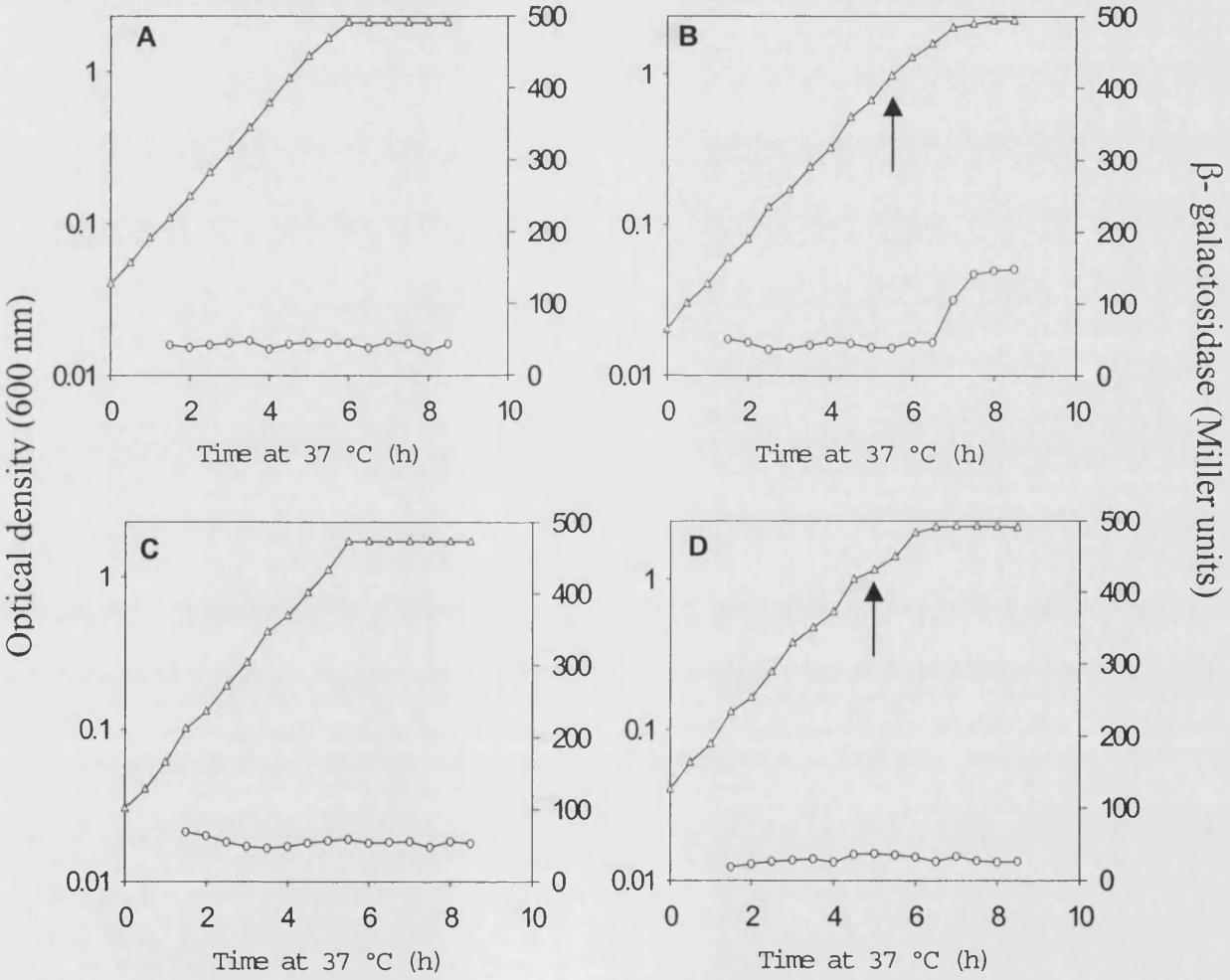


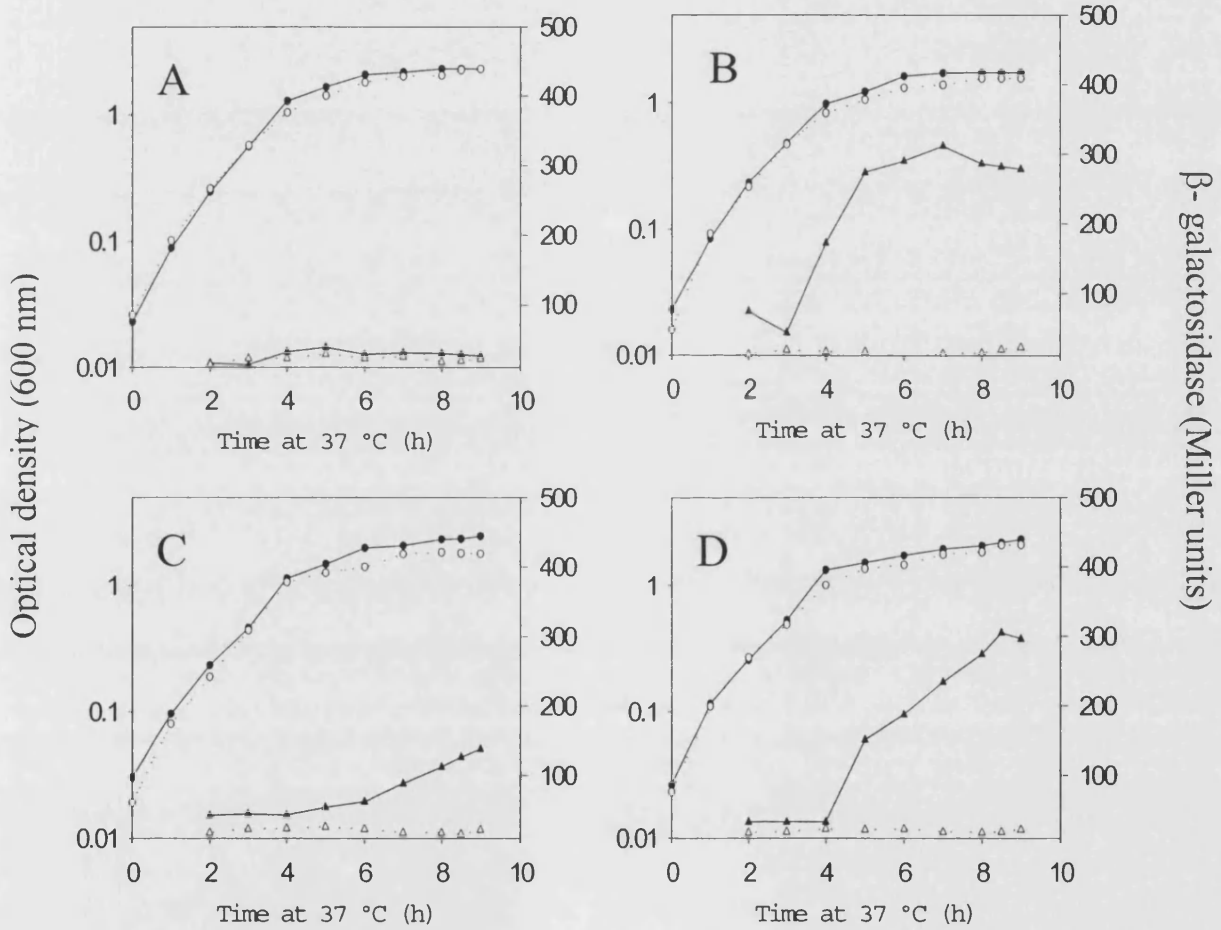
Figure. 3-19. Induction of *rpoS742::lacZ* in response to temperature switch from 37 to 30 °C for planktonic *E. coli* RO91 (late-translational fusion) subjected to no temperature shift (A), temperature shift (B) and *E. coli* RO200 (transcriptional fusion) subjected to no temperature shift (C), temperature shift (D). (Temperature shift is represented by an arrow).



3.2.13 The influence of the stringent response on *rpoS* induction under conditions of nutrient limitation

The stringent response is known to regulate *rpoS* positively at the transcriptional level (Gentry et al., 1993) and in an uncharacterised manner via inorganic polyphosphate (Shiba et al., 1997). The stringent response also provides a link between growth rate and *rpoS* expression as the production of the alarmone (p)ppGpp is directly connected to growth rate (Teich et al., 1999; Chatterji and Kumar Ojha, 2001). Therefore, it was decided to examine the influence of the stringent response on *rpoS* induction under conditions of nutrient limitation. Phage transduction (P1vir) was used to produce a *relA* knockout in the *rpoS* late-translational reporter strain (RO91). *RelA* was transduced from the donor strain CF1693 into RO91 to make AWS91 (RO91Δ *relA*). Attempts were made to construct a *spoT* deletion but these proved unsuccessful. The expression of *rpoS* under conditions of nutrient limitation was examined using AWS91 in high-density planktonic culture. It was found that deletion of *relA* completely abolished *rpoS* expression and no induction was seen in response to any of the limitations tested (fig. 3-20.). The RelA deleted strain proved to be auxotrophic for amino acids and so the CDM_{10} had to be supplemented with casamino acids (0.1%). This concentration was chosen as it was shown to be the lowest that enabled growth to the same density as the control (RO91). However, the addition of casamino acids to a chemically defined medium was not ideal as it is completely undefined and had an effect on nutrient limitation. A slight increase in *rpoS* expression was seen in the control (RO91) in response to nitrogen limitation when CDM_{10} contained casamino acids. It is likely that this occurred because the cells were no longer truly nitrogen-limited and were readily using casamino acids as a supplementary nitrogen source.

Figure. 3-20. Influence of RelA on *rpoS742::lacZ* translational fusion induction (triangles) throughout the growth cycle (circles) for high density planktonic *E. coli* RO91 (filled symbols) and AWS91 (RO91 Δ *relA*) (open symbols) grown in CDM_{10} with carbon (A), magnesium (B), nitrogen (C) and phosphate (D) limitation at 37 °C with aeration.



3.3 DISCUSSION

3.3.1 Expression of rpoS in response to nutrient limitation

To date, entry into stationary phase was assumed to result in a concurrent increase in *rpoS* expression. However, this study has produced the novel finding that entry into stationary phase does not invariably lead to *rpoS* induction. It was found that under conditions of carbon and nitrogen limitation, entry into stationary phase occurred without *rpoS* induction in planktonic culture. These results were obtained using LacZ reporter constructs and have been confirmed by the examination of downstream gene expression (*uspB*) and by Western blot analysis. However, these results are extremely surprising, as a number of previous studies have reported *rpoS* expression in response to carbon limitation (Zgurskaya et al., 1997; Lange and Hengge-Aronis, 1994). The response to nitrogen limitation has not been widely studied. Previous studies have found that during late exponential phase *rpoS* translation is stimulated and at the onset of stationary phase, translation is reduced and the turnover of σ^S is inhibited. A continuous increase in σ^S concentration from late exponential until at least 5 hours into stationary phase has also been reported (Lange and Hengge-Aronis, 1994).

There are a number of possible explanations for the discrepancy between this and previous studies. It is important to note that this study employed a defined medium in which it was known that all nutrients (except the single limiting nutrient) were in excess such that the complete medium was capable of supporting growth to a theoretical optical density (600 nm) of 10. Previous studies have used poorly defined minimal media (based on M9 medium Miller, 1972) to achieve carbon limitation (Zgurskaya et al., 1997; Lange and Hengge-Aronis, 1994). It is feasible that in M9 medium entry into stationary phase is not solely a result of carbon limitation and that

it may be due to a combination of factors. Iron is not routinely added to M9 medium and if high-quality deionised water / ultrapure chemicals are used in preparation, it is conceivable that inherently low iron levels may restrict the growth of *E. coli* in this medium. Examining *rpoS* expression in M9 medium with and without additional iron tested this hypothesis. It was found that M9 medium prepared without additional iron induced high-level *rpoS* expression whereas if iron was replete, there was no induction of *rpoS*. These results are slightly surprising as it was previously thought that *E. coli* had such a low requirement for iron that the trace quantities of contaminating iron present in the medium would be sufficient to support growth. Additionally, one study that reports *rpoS* induction in response to carbon limitation used a glucose concentration that is not limiting (0.3 %) (Zgurskaya et al., 1997). Under these conditions, it is likely that stationary phase results from other factors, probably a combination of oxygen, iron and carbon limitation and it is not surprising that *rpoS* induction occurs in response to the multitude of combined stresses.

The high *rpoS* levels that were reported at least 5 hours after the onset of stationary phase in a previous study (Lange and Hengge-Aronis, 1994) could have resulted from a drop in pH. It was found in this study that in M9 with glucose as a carbon source the pH of the medium had decreased by more than one pH unit 6 hours into stationary phase (results not shown). The CDM_{10} used in this study has a greater buffer capacity than M9 and no pH drop was found even in cultures that were 18 hours into stationary phase.

3.3.1.1 Influence of growth rate on the induction of rpoS

The results obtained with planktonic cultures in this study seem to indicate that the rate of change in growth rate that occurs as cells enter stationary phase may influence *rpoS* induction. Nutrient limitations that induced *rpoS* (magnesium and phosphate) resulted in a gradual slowing of growth rate as the cells entered stationary phase. Conversely, carbon and nitrogen limitation caused a sudden cessation of growth with no detectable slowing of growth rate upon entry into stationary phase. It was therefore proposed that *rpoS* induction occurs only if there is a change in growth rate before entry into stationary phase. In order to test this hypothesis cells were grown in CDM_{10} (with a limiting concentration of carbon) and the growth rate perturbed to determine if this would cause *rpoS* induction.

RpoS induction was found to occur if cells were switched from 37 to 30 °C or from 200 to 0 rpm (at a low cell density) with a corresponding slowing of growth rate. However, this by no means proves that a decrease in growth rate is necessary for *rpoS* expression as other factors may have contributed to *rpoS* induction under these circumstances. It is known that low temperature causes *rpoS* induction by the expression of a small RNA, *dsrA* (Sledjeski et al., 1996). *DsrA* increases *rpoS* translation by stabilising *rpoS* mRNA, preventing the formation of hairpin loops (Majdalani et al., 1998). It is feasible that low aeration itself may also influence *rpoS* induction. Therefore, it is impossible to distinguish under these circumstances whether *rpoS* induction results from the change in growth rate or if lower temperature / aeration may itself be stressful to the cell and cause *rpoS* induction. To determine unambiguously the influence of growth rate on *rpoS* induction chemostat studies would be necessary. Unlike batch culture, in which the different stages of the

decelerating phase (prior to stationary phase) merge into each other and are therefore difficult to study, chemostat culture enables isolation of each component stage in a time-independent manner. A number of studies have examined *rpoS* induction in chemostat culture. However, as with many other studies the medium used was poorly defined, probably causing iron restriction and inadequately buffered (Liu et al., 2000; Tweeddale et al., 1998; Notley and Ferenci, 1996). Nevertheless it has been demonstrated in carbon-limited, steady state chemostat culture that low growth rate (with a doubling time of 3.5 h or longer) is necessary for high-level *rpoS* induction and that *rpoS* induction does not occur if growth is rapid (Notley and Ferenci, 1996). It is feasible that under conditions of slow growth the stringent response is induced leading to positive regulation of *rpoS*.

3.3.1.2 Role of the stringent response in the induction of rpoS

The influence of the stringent response, notably the alarmone (p)ppGpp on *rpoS* expression in response to nutrient limitation was examined. The alarmone (p)ppGpp provides a direct link between starvation and growth rate in the stressed *E. coli* cell (Teich et al., 1999; Chatterji and Kumar Ojha, 2001). It is already known that (p)ppGpp is essential for *rpoS* induction at the transcriptional level and in an uncharacterised manner via inorganic polyphosphate (Shiba et al., 1997; Gentry et al., 1993).

A *relA* null mutant was produced in the late-translational *lacZ* reporter (R091), a MC4100 background. It was found that *rpoS* induction was abolished under all nutrient limitations in this strain. This is interesting to note as the other gene capable of ppGpp production, *spoT,* was left intact. This indicates that *spoT* has a negligible

contribution to ppGpp production in the strain used in the conditions of this study. This is slightly surprising as it is known that (p)ppGpp production under conditions of carbon and phosphate limitation normally occurs via *spoT* (Cashel, 1994 ; Gentry and Cashel, 1996; Murray and Bremer, 1996) (to date, magnesium and nitrogen limitation have not been studied). However, the precise genotype of the strain MC4100 may be responsible for this anomalous result. It is known that the MC4100 *relA* and *spoT* genes are partially inactive and it has been suggested that the ppGpp synthetic activity of MC4100 *spoT* may be much lower than usual (Cashel, personal communication, 2001). It is possible that the intrinsically low activity of MC4100 *spoT* has resulted in compensation by *relA*. Therefore, when *relA* is deleted, all ppGpp activities are lost including *rpoS* induction. It would be desirable to repeat this study using a different strain background, preferably one with 'normal' *relA*/*spoT* activity.

Indirectly, the stringent response and associated activities may help to explain the absence of *rpoS* expression under conditions of carbon limitation. As discussed previously, high levels of ppGpp induced in response to nutrient limitation normally lead to the production of large quantities of inorganic polyphosphate. Inorganic polyphosphate is required for *rpoS* induction / activity and crucially, inorganic polyphosphate is not produced in response to carbon limitation although there are high levels of ppGpp (Ault-Riché et al., 1998; Kornberg et al., 1999). It is feasible that the absence of inorganic polyphosphate under conditions of carbon limitation represses *rpoS* induction. However, the same explanation cannot be applied to conditions of nitrogen limitation as inorganic polyphosphate is significantly elevated under these conditions (Ault-Riché et al., 1998; Kornberg et al., 1999).

3.3.1.3 Effect of nitrogen limitation on rpoS induction: the role of specific stress responses

It is more difficult to find a specific explanation for the absence of *rpoS* under nitrogen limitation. It is feasible that *rpoS* is not induced in response to nitrogen limitation because there is already a specific stress response for nitrogen limitation. The nitrogen starvation-associated sigma factor σ^{54} is induced in response to nitrogen limitation and directs RNA polymerase to transcribe a set of genes specific for nitrogen starvation. *E. coli* is thought to contain approximately 30 σ^{54} dependent promoters, most of which are involved in nitrogen metabolism and scavenging (Reitzer and Schneider, 2001).

The idea that a general stress response may be forfeited in favour of a more specific response is not novel. The small RNA *oxyS* is induced in response to oxidative stress and inhibits *rpoS* translation (Altuvia et al., 1997). As *oxyS* is induced in response to hydrogen peroxide it has been proposed that this mechanism prevents the unnecessary induction of σ^S when the oxidative stress response alone would suffice. Therefore, it is possible that the general stress response is not induced in response to nitrogen limitation as a specific response to nitrogen limitation already exists. As the concentration of RNA polymerase is relatively constant (Jishage et al., 1996), if large quantities of σ^S were produced it would lead to competition between σ^S and σ^{54} for the available RNA polymerase. σ^{54} is only present at a very low level (approximately 20 molecules per cell in MC4100 under conditions of ammonia starvation) (Jishage et al., 1996). Therefore, if σ^S was upregulated to a high level there would be little chance of σ^{54} binding to RNA polymerase and transcribing nitrogen-starvation associated genes.

3.3.1.4 Effect of carbon and nitrogen limitation on nutrient transport systems

There is one explanation that can rationalise the lack of *rpoS* expression in response to both carbon and nitrogen limitation. A recent chemostat study has examined the physiology of so called ''hungry'' cells (Ferenci, 2001). The term 'hungry' is used by the author to differentiate between cells that have stopped growing because of nutrient limitation, and cells that are growing very slowly and utilising nanomolar concentrations of nutrients via high affinity nutrient uptake and scavenging pathways (Ferenci, 2001). Specifically, this term is used to describe chemostat cells that are growing very slowly with limiting concentrations of carbon or nitrogen. If cells were 'hungry' it was found that *rpoS* was detrimental as it has a strong, negative effect on the expression of high-affinity carbon and nitrogen nutrient transport pathways. Intriguingly, it was even found that there was strong selection for drastic loss-of-function mutations in *rpoS* (Ferenci, 2001). Therefore, it seems reasonable that *rpoS* mutation or lack of expression is favourable under carbon and nitrogen starvation, as it would enable the utilisation of any remaining nutrients and allow growth to continue for a longer period.

3.3.2 Influence of nutrient limitation on the transcription of rpoS

This study also examined the contribution of increased transcription of *rpoS* to cellular σ^S levels under conditions of nutrient limitation. No increase in *rpoS* transcription (as determined using a late-transcriptional *lacZ* reporter fusion, strain R0200) was found under any of the nutrient limitations tested. This is not entirely surprising, as previous studies have detected no increase or even a decline in transcription in response to entry into stationary phase (Zgurskaya et al., 1997). It is known that the cellular level of σ^S increases predominantly because of increased

stability. Previously, no explanation has been proposed for the decrease of *rpoS* transcription (Zgurskaya et al., 1997). However, there is a reasonable explanation for the inhibition of *rpoS* transcription under certain conditions of starvation, especially carbon limitation. It is known that in response to carbon exhaustion a burst of cAMP production and therefore a strong and rapid increase in intracellular cAMP levels results (Ishizuka et al., 1993). Many carbon starvation associated genes are positively regulated by cAMP/cAMP receptor protein (CRP) and show induction under these conditions (Ishizuka et al., 1993). However, *rpoS* is negatively regulated by cAMP-CRP at the transcriptional level (Hengge-Aronis, 1996) and so it could be expected that conditions of carbon limitation could (at least transiently) inhibit *rpoS* transcription.

3.3.3 Influence of growth as a biofilm on the induction of rpoS

When *rpoS* induction was examined in the biofilm model, the pattern of induction was strikingly different from planktonic culture. In biofilm-grown cultures, *rpoS* was found to be strongly expressed under all of the nutrient limitations tested. These results are not entirely surprising as it has long been known that attached cells exhibit a distinctly different physiological state to free-floating, planktonic cells, even (as in this study) if cells are grown in the same medium (Brown and Gilbert , 1993; Brown et al., 1988). The different physiological status of biofilms has been proposed to explain their persistence and resistance to antimicrobial agents. It has been proposed that biofilm growth may lead to an early and more complete general stress response compared with planktonic cells (Brown and Barker, 1999). It has been suggested that slow growth rate and high cell density leads to *rpoS* induction and interestingly, these are two characteristics typical of biofilms. The biofilms used in this study were

certainly seen to exhibit a slower growth rate than their corresponding planktonic counterparts and it is feasible that this may have contributed to *rpoS* induction seen even in the carbon- and nitrogen-limited cells. Biofilms are also, by definition, of high cell-density as the cells are attached and in very close proximity to one another. However, in *E. coli* the contribution of cell density to *rpoS* induction seems negligible (as discussed below).

Although the method used in this study provides the best means of producing reproducible, nutrient-limited biofilms it is important to be aware of the limitations of this system. The biofilms grown did not realistically represent biofilm growth in the natural environment as there was no aqueous interface. As a result of this the system lacks a dynamic equilibrium and there is no interaction or exchange between planktonic and attached cells. However, despite these points this system represents a method for producing biofilms that are directly comparable to their planktonic counterparts (that is, subject to the same nutrient limitation).

It is relatively difficult to compare the results obtained in this study with those published previously as there are few examples of analysis of *rpoS* in *E. coli* biofilms. The only study of *rpoS* in *E. coli* biofilms to date, used a poorly defined medium with 0.025% glucose (a potentially limiting concentration). The level of *rpoS* induction was comparable to the level in this study under carbon limitation but unlike this study, their planktonic culture also exhibited *rpoS* expression (Adams and McLean, 1999). Most other studies of *rpoS* in biofilms have involved *P. aeruginosa*. Recently, it has been suggested that *rpoS* may strongly influence biofilm physiology in *P. aeruginosa* as *rpoS* mutants exhibited thicker and more complex biofilms compared with their

wild type parents. Additionally, *rpoS* expression was lower in biofilm compared with planktonic culture (Whiteley et al., 2001). However, there are a number of important points that must be considered. The study used microarray analysis to examine *rpoS* mRNA expression, although it is well documented that σ^S is under complex translational and post-translational control (Lange and Hengge-Aronis, 1994). Also, the study did not use completely chemically defined media and the biofilm/planktonic cells were not produced in a comparable manner.

It is apparent that there is great scope for future work examining the role of *rpoS* in biofilm formation and physiology. It would be particularly interesting to use GFP-based reporters and scanning confocal laser microscopy. This technique would enable examination of the heterogeneous nature of the biofilm population as the gene expression of single cells at different locations within the biofilm could be analysed. This could be extremely interesting and would not be achievable using *lacZ* based reporter strains. One of the inherent limitations present in using *lacZ* reporter based analysis is that if the cells are not examined *in situ* any data obtained represent an average of the whole population and cannot reflect gene expression at the level of the individual cell.

3.3.4 Role of cell density in the induction of rpoS

This study also examined the influence of population density on *rpoS* expression. It was found that population density had no influence on *rpoS* expression under any of the conditions tested. Only one previous study has alluded to the influence of population density on *rpoS* expression in batch culture. It was found that there was no *rpoS* expression if cells were carbon-limited at an OD (600nm) of 0.1 (Lange and

Hengge-Aronis, 1994). However, it is likely that this finding reflects 'true carbon limitation' where, as in this study there is no *rpoS* expression and that the 'high-density' finding (with high *rpoS* induction) in their study was partly a result of low iron concentration. It is feasible that even if no iron were added to the medium there would be enough contaminating iron present to support growth to a 'low' cell density.

Previous studies have examined cell density in chemostat culture where it was found that there was an eight-fold increase in *rpoS* levels between bacteria grown at 10^8 and 10^9 cells/ml (Liu et al., 2000; Tweeddale et al., 1998). There is no direct evidence for the link between high cell density-dependent events (i.e. quorum sensing) and *rpoS* induction in *E. coli*. This is unsurprising as to date only the LuxS-regulated quorum sensing mechanism has been identified (Surette and Bassler, 1998). As LuxS-mediated events are maximal in mid-log and decrease upon entry into stationary phase it is difficult to see how it could positively regulate *rpoS* expression (Surette and Bassler, 1998; Surette et al., 1999). A link between quorum sensing and *rpoS* has been identified in *P. aeruginosa* but this is mediated by AHLs (which are not present in *E. coli*) (Whiteley and Parsek, 2000).

To conclude, this study has shown that entry into stationary phase is not always concurrent with *rpoS* expression in planktonic culture. This is not altogether surprising, as it is known that cells can survive in the absence of *rpoS*. In fact under certain laboratory conditions, *rpoS* mutations are common and this situation has been mirrored in environmental isolates where loss of *rpoS* function is widespread and advantageous to survival (Zambrano et al., 1993; Jishage and Ishihama, 1997). It will be interesting to determine the effect of specific nutrient limitation and *rpoS* on the

stationary phase phenotype and the response to stress. This will be will be examined in the following chapters.

4 EFFECT OF *RPOS* AND NUTRIENT LIMITATION ON PHYSIOLOGICAL CHANGES DURING STATIONARY PHASE

4.1 INTRODUCTION

Stress, including that of nutrient limitation, high osmolarity, low pH and suboptimal temperature can result in the slowing of growth or entry into stationary phase. Physiological consequences of stationary phase include: resistance to multiple stresses (Lange and Hengge-Aronis, 1991; Hengge-Aronis, 1996) and structural changes such as, accumulation of storage compounds, protection and condensation of the DNA and alteration of the cell envelope (reviewed in (Huisman et al., 1996; Lange and Hengge-Aronis, 1991)). Many of these phenotypic changes are regulated by σ^S. In addition, stationary phase is typically associated with high population density. Population density is known to regulate coordinated changes in gene expression via quorum sensing (Bassler, 1999).

4.1.1 Trehalose

Trehalose is a non-reducing disaccharide of glucose that rapidly accumulates in the periplasm in response to high salt (Garcia De Castro et al., 2000) heat shock and at the onset of stationary phase (Hengge-Aronis et al., 1991). Trehalose is a compatible solute and has a protective effect on proteins and biological membranes during desiccation, high osmolarity, freeze-thaw and heat shock (De Smet et al., 2000) (Lange and Hengge-Aronis, 1991). The synthesis of trehalose is regulated by *rpoS* via the genes *otsA* and *otsB* which encode a trehalose-6-phosphate synthase and a trehalose-6-phosphate phosphatase, respectively (Horlacher et al., 1996). Trehalose

may also be utilized as a carbon source if it is no longer required for stress protection by the σ^S -regulated, cytoplasmic trehalase *treF* (Horlacher et al., 1996).

The separate influences of *rpoS*, population density and nutrient limitation have not previously been elucidated. In addition, the influence of biofilm culture on trehalose production has not been studied.

4.1.2 Cyclopropane fatty acid production

Changes in the cell envelope are significant as they occur at the interface between the cell and its environment and may act to minimize the effects of environmental stress on the rest of the cell. One major physiological change involves the production of cyclopropane fatty acids. Cyclopropane fatty acid (CFA) formation is a post-synthetic modification of the lipid bilayer that occurs as *E. coli* enters stationary phase (Wang and Cronan, 1994; Grogan and Cronan, 1997). The reaction is catalyzed by CFA synthase, a soluble enzyme that is under the control of two promoters. The distal promoter P1 is a standard σ^{70} promoter that is active throughout the growth cycle whereas the proximal P2 promoter is σ^S specific and active only during the transition into stationary phase (Wang and Cronan, 1994). It is thought that CFA formation may contribute to bacterial survival during stationary phase. CFAs, unlike unsaturated fatty acids, may protect against certain forms of oxidation and assist survival under hyperbaric conditions (Grogan and Cronan, 1997). CFA formation is implicated in resistance to 20 % ethanol (vol / vol), freeze-thaw treatments (Grogan and Cronan, 1986) and rapid pH drop (Chang and Cronan, 1999; Brown et al., 1997). The correlation between resistance to acid shock (pH 3) and CFA content has been

proposed to be due to decreased proton permeability or increased active proton efflux (Chang and Cronan, 1999).

4.1.3 LuxS-mediated quorum sensing

Quorum sensing in *E. coli* is reminiscent of the two component signal transduction networks that are utilised for quorum sensing in Gram positive bacteria. The production of autoinducer (AI-2) molecule is dependent on the *luxS* gene (Surette et al., 1999) (Surette and Bassler, 1998). The precise structure of AI-2 is unknown but it is proposed to be a furanone (Schauder et al., 2001). Unlike acylated homoserinelactone (AHL) mediated quorum sensing, LuxS-dependent quorum sensing is thought to enable communication within and between species (Surette et al., 1999). Previous studies have shown that AI-2 production interacts with other stress responses. Links between LuxS-dependent quorum sensing and the SOS response (Sperandio et al., 2001) and the σ^{32} mediated cytoplasmic heat shock response (DeLisa et al., 2001) have been documented. However, the influence of *rpoS* on LuxS-mediated quorum sensing has not been established.

4.2 RESULTS

4.2.1 Effect of *rpoS* and nutrient limitation on trehalose production

Trehalose levels were measured by assaying the release of free glucose following incubation of samples with a porcine trehalase. Glucose was measured using an enzymatic glucose oxidase/peroxidase assay with *o*-dianosidine as the substrate. Trehalose was measured for planktonic and biofilm, stationary phase (6 hours after entry) *E. coli* RH90 (Δ*rpoS*) and MC4100. Cells were grown in CDM_{10} with carbon, magnesium, nitrogen and phosphate limitation. Both low- and high-density

planktonic cells were assayed for trehalose. Only high-density biofilm cells were assayed, since the large number of cells required made it impractical to assay low-density biofilm culture. The results were corrected for free glucose observed in the absence of trehalase treatment (fig. 4-1, 4-2).

Under all conditions tested more trehalose was produced in the wild type (MC4100) compared with the *rpoS* null mutant (RH90). However, this was more pronounced in the planktonic than the biofilm cultures. No density-dependent effects were observed in planktonic culture; both low and high-density cultures showed similar patterns of trehalose production. In planktonic MC4100, very little trehalose was produced in cells subjected to carbon limitation (less than 0.15 μg trehalose/ 1.25 x 10^{10} cells), nitrogen-limited cells had more trehalose (around 1.8 μg trehalose/ 1.25 x 10^{10} cells), magnesium-limited cells had an even greater concentration (2.5 to 3 μg trehalose/ 1.25 x 10^{10} cells) and phosphate-limited cells exhibited the highest concentration of all (approximately 3.5 μg trehalose/ 1.25 x 10^{10} cells). The *rpoS* mutant (RH90) exhibited very low trehalose (less than 0.5 μg trehalose/ 1.25 x 10^{10} cells) under conditions of carbon, magnesium and phosphate limitation. However, in response to nitrogen limitation, trehalose levels were elevated (over 1.1 μg trehalose/ 1.25 x 10^{10} cells) even in the absence of *rpoS*.

When cells were grown as a biofilm, the level of trehalose produced in the absence of *rpoS* (RH90) was at least 3 times greater compared with the corresponding planktonic culture. Overall, higher levels of trehalose were observed in the biofilm compared with planktonic culture and the pattern of expression was slightly different. In both biofilm and planktonic culture, phosphate-limited cells had the highest level of

trehalose and carbon-limited cells had the lowest level of trehalose. In the biofilm, wild type nitrogen-limited cells had greater trehalose than magnesium-limited cells, whereas the reverse was observed in planktonic cells (fig. 4-2).

Figure 4-1. Effect of nutrient limitation on trehalose production for stationary phase (6 hours after entry) planktonic *E. coli* MC4100 and RH90 (Δ *rpoS*) grown in carbon-, magnesium-, nitrogen- and phosphate-limited CDM_{10} to low- and high-density at 37 °C with aeration.

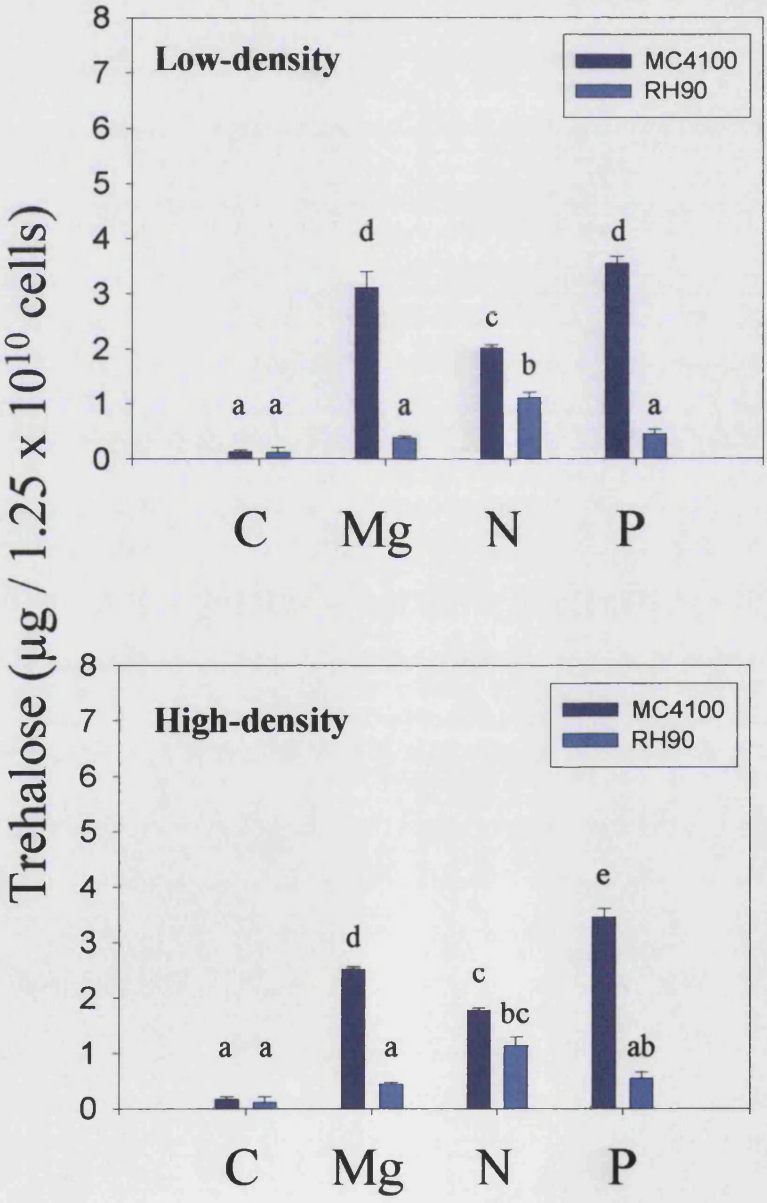
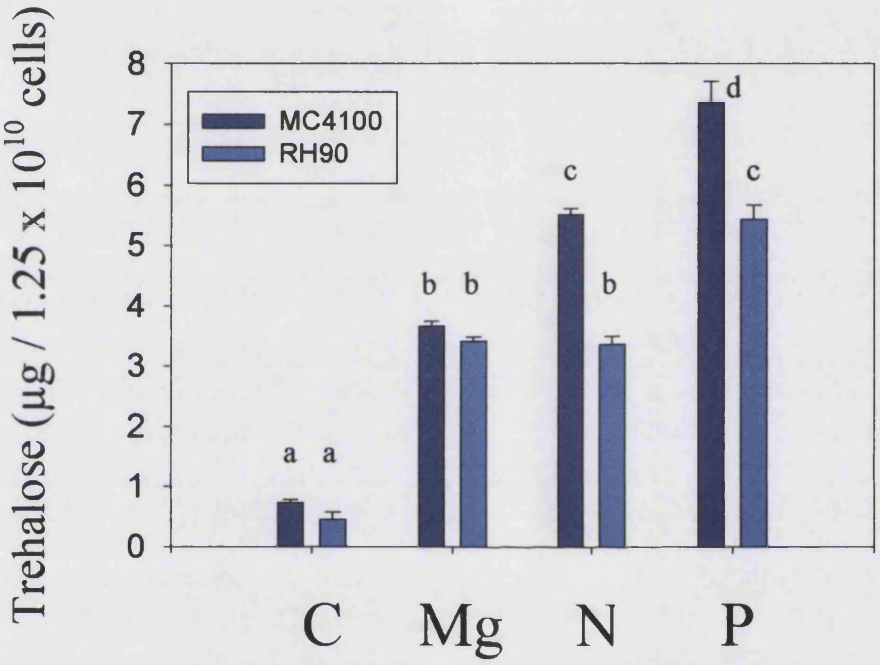


Figure 4-2. Effect of nutrient limitation on trehalose production for stationary phase (6 hours after entry) biofilm *E. coli* MC4100 and RH90 (Δ *rpoS*) grown on carbon-, magnesium-, nitrogen- and phosphate-limited CDM_{10} at 37 °C.



4.2.2 Effect of nutrient limitation on the fatty acid profile of planktonic and biofilm *E. coli* MC4100 and RH90 (Δ *rpoS*)

Fatty acid methyl ester (FAME) analysis was used to determine the complete fatty acid profiles of nutrient-limited *E. coli* MC4100 and RH90. Stationary phase (6 hours after entry) carbon-, magnesium-, nitrogen- and phosphate-limited cells were analyzed from planktonic and biofilm cultures. Both low- and high-density stationary phase planktonic cultures, biofilm stationary phase cultures and log-phase high-density planktonic culture were examined. In total over 36 individual fatty acids were detected. The amount of each fatty acid is presented as the percentage relative abundance within the total fatty acid composition of that particular sample. Cluster analysis was undertaken to determine the relationships between fatty acid profiles of *E. coli* strains RH90 and MC4100 grown under nutrient limitation. Euclidean distances were calculated between pairs of samples to produce a dissimilarity matrix. Dendrograms were constructed using average linkage between groups (SPSS for Windows). Short branch lengths separate similar fatty acid profiles, longer branches indicate greater differences between profiles (fig. 4-3, 4-4, 4-5). Samples that were separated by less than 10 Euclids were clustered together (blue labels). According to this classification the high-density planktonic samples were separated into 4 groups. The carbon, magnesium and nitrogen log samples and the stationary phase carbon-limited samples were grouped together. A second group comprises the phosphate log samples and the stationary phase magnesium and nitrogen samples. The other two groups present in high-density planktonic culture were that of stationary phase phosphate-limited RH90 and MC4100 (fig. 4-3). Stationary phase low-density planktonic cells were divided into 4 groups (log phase cells were not analyzed). The 4 groups were the same as for high-density planktonic culture. Nitrogen- and

magnesium-limited cells were placed together in one group, carbon-limited cells in a second group, a third group of RH90 phosphate-limited cells and a fourth of MC4100 phosphate-limited cells (fig. 4-4). Biofilm cultured cells exhibited considerably different fatty acid profiles compared with their planktonic counterparts. A distance of 10 Euclids could separate biofilm cells into 3 groups. Stationary phase carbon-limited cells and MC4100 magnesium-limited cells comprise one group, nitrogen-limited cells and RH90 magnesium-limited cells another group and the third group contains phosphate-limited MC4100 and RH90 (fig. 4-5). It is interesting to note that according to FAME literature, 2 Euclids is the maximum deviation within a strain group, 6 Euclids within a species group and 10 Euclids within a genus group (Sasser, 1990). Based on current bacterial identification techniques using FAME analysis, nearly all of the samples would be assigned to different species and many would be placed in different genera. Therefore, these results indicate that nutrient limitation and *rpoS* have a dramatic effect on the fatty acid composition of *E. coli* MC4100 and RH90.

Cyclopropane fatty acids were identified and the percentage of total fatty acids were determined (fig 4-6). The majority of CFAs were of the 17-carbon type (c-17) however, a small proportion of 19-carbon (c-19) were detected. The total percentage of CFAs presented in fig 4-6 is the sum of c-17 and c-19 CFAs.

The overall pattern of CFA abundance in nutrient-limited cells was examined and it was found that carbon-limited cells had the least CFAs, phosphate-limited the greatest levels and magnesium and nitrogen-limited cells had intermediate levels. Under all culture conditions the *rpoS* null mutant had less CFAs than the wild type strain

(MC4100). In planktonic culture the overall pattern of expression was essentially similar regardless of the cell density. The only apparent difference between high and low density cells was a marginally elevated level of CFAs in the high density phosphate-limited culture compared with the equivalent low density culture. In planktonic culture very low levels of CFAs were detected in carbon-limited cells (less than 3 %), magnesium and nitrogen-limited cultures exhibited below 12 % CFAs whereas the level in phosphate-limited cells varied between 26 and 40% depending on the cell density. The standard error between samples was calculated and is shown as error bars.

Biofilm cells were analyzed for the presence of CFAs and found to contain similar levels to the corresponding planktonic cultures (fig.4-7). The overall level of CFAs was slightly elevated in carbon-, magnesium- and nitrogen-limited biofilms compared with their planktonic counterparts. In particular, carbon-limited cells showed significantly increased CFA content (up to 10 %) whereas only negligible levels were detected in planktonic culture. Magnesium- and nitrogen-limited MC4100 cells had somewhat higher CFA content (up to 15 %) compared with the equivalent planktonic culture. Phosphate-limited biofilms exhibited similar CFA levels to planktonic culture. Overall, the pattern of CFA content was remarkably similar to that obtained for trehalose.

Figure 4-3. Dendrogram illustrating the influence of nutrient limitation on the fatty acid profile of planktonic high-density *E. coli* MC4100 and RH90 (Δ *rpoS*) grown in CDM_{10} at 37 ° C with aeration. Based on the Euclidean distance between pairs of samples and clustered using the unweighted pair group method with arithmetic averages (UPGMA) (where C, M, N and P represent carbon-, magnesium-, nitrogen- and phosphate limited cells, + and - refer to the presence or absence of *rpoS* and log and stat refer to log- and stationary phase cultures, respectively).

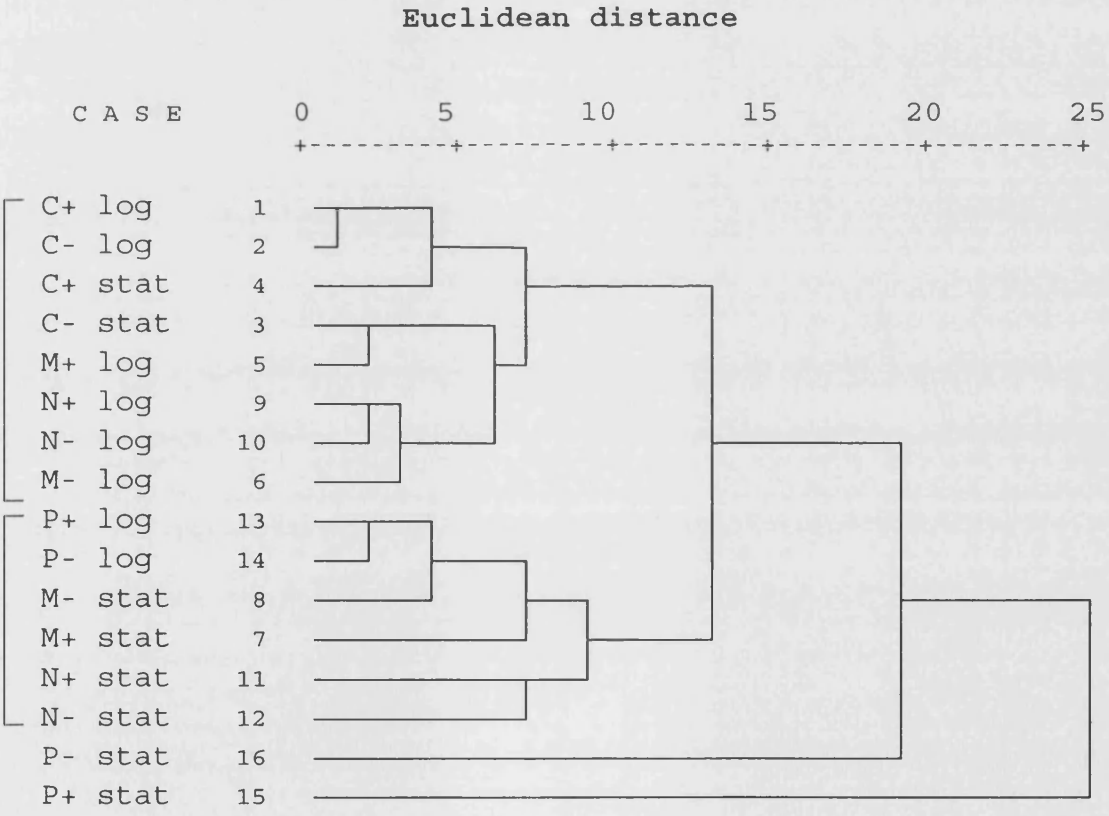


Figure 4-4. Dendrogram illustrating the influence of nutrient limitation on the fatty acid profile of planktonic low-density *E. coli* MC4100 and RH90 (Δ *rpoS*) grown in CDM_{10} at 37 ° C with aeration. Based on the Euclidean distance between pairs of samples and clustered using the unweighted pair group method with arithmetic averages (UPGMA) (where C, M, N and P represent carbon-, magnesium-, nitrogen- and phosphate limited cells, + and - refer to the presence or absence of *rpoS* and log and stat refer to log- and stationary phase cultures, respectively).

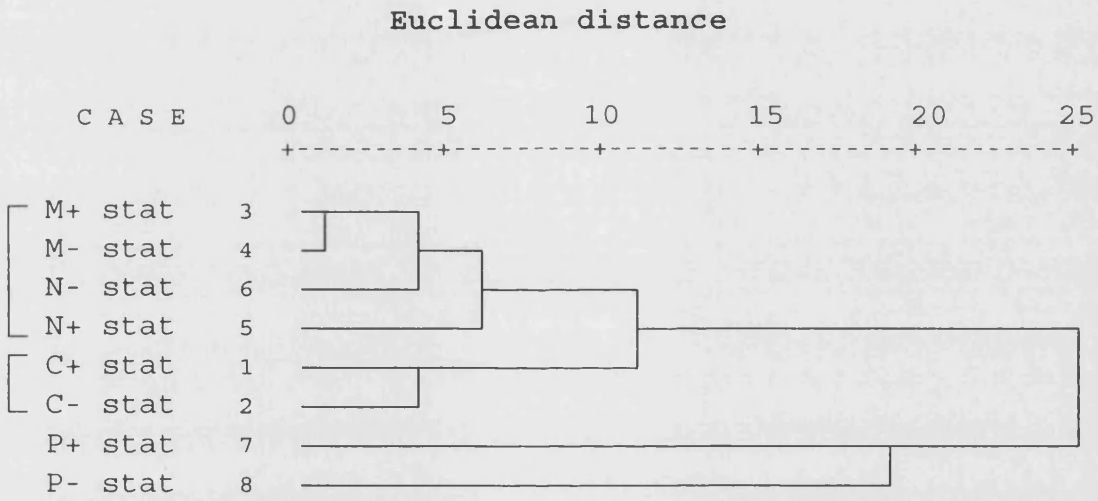


Figure 4-5. Dendrogram illustrating the influence of nutrient limitation on the fatty acid profile of biofilm *E. coli* MC4100 and RH90 (Δ *rpoS*) grown on CDM_{10} at 37 ° C. Based on the Euclidean distance between pairs of samples and clustered using the unweighted pair group method with arithmetic averages (UPGMA) (where C, M, N and P represent carbon-, magnesium-, nitrogen- and phosphate limited cells, + and - refer to the presence or absence of *rpoS* and log and stat refer to log- and stationary phase cultures, respectively).

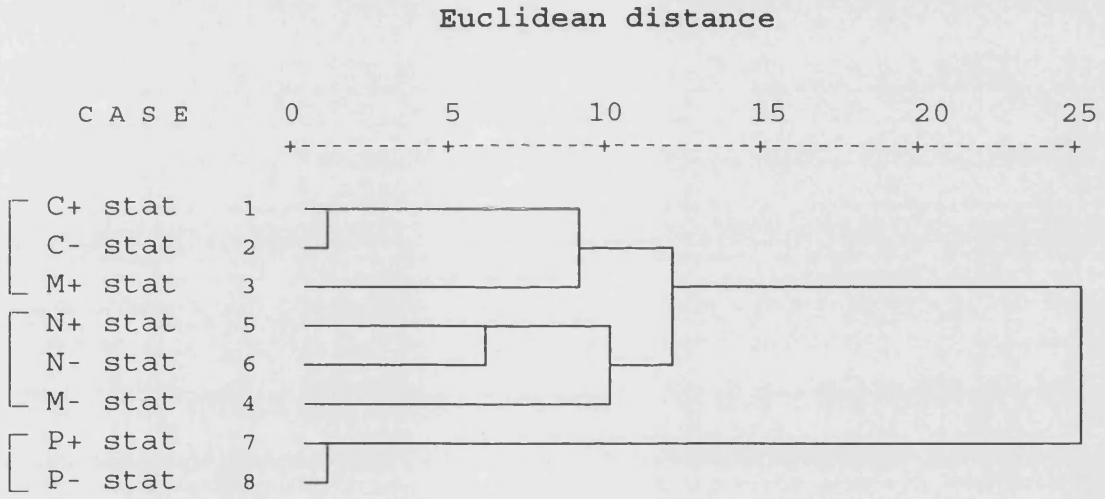


Figure 4-6. The effect of nutrient limitation on abundance of CFAs in stationary phase (6 hours after entry) planktonic *E. coli* MC4100 and RH90 (Δ *rpoS*) grown in carbon-, magnesium-, nitrogen- and phosphate-limited CDM_{10} to low- and high-density at 37 °C with aeration.

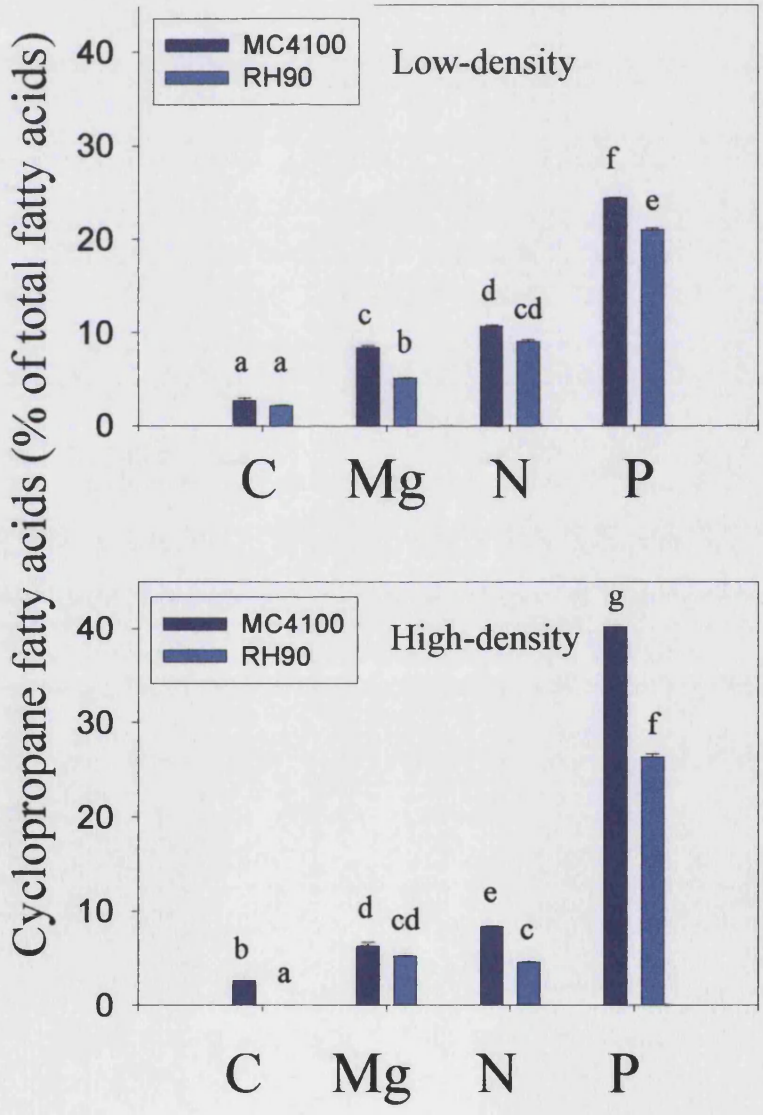
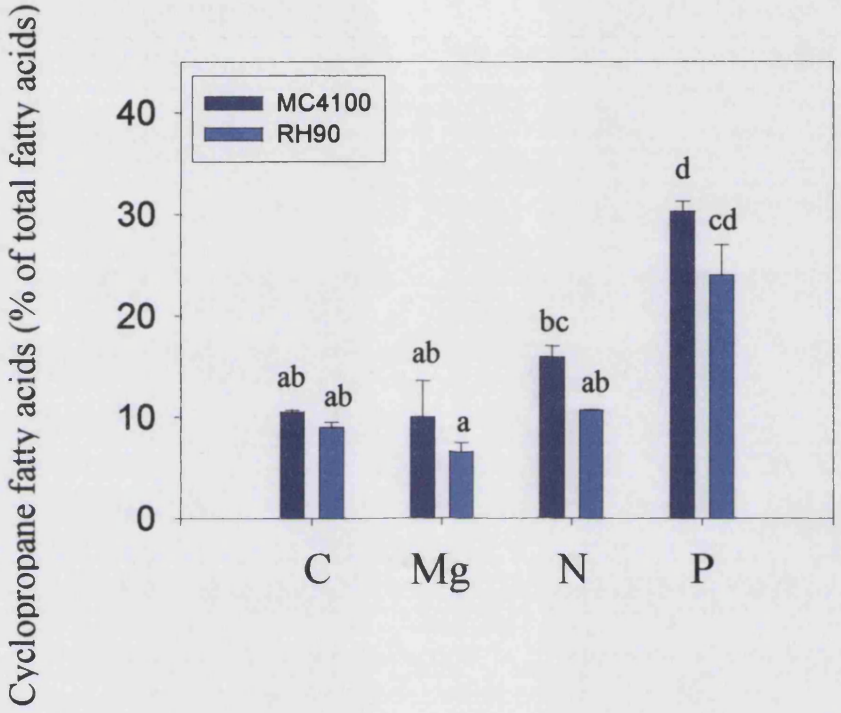


Figure 4-7. The effect of nutrient limitation on abundance of CFAs in stationary phase (6 hours after entry) biofilm *E. coli* MC4100 and RH90 (Δ *rpoS*) grown on carbon-, magnesium-, nitrogen- and phosphate-limited CDM_{10} at 37 °C.



4.2.3. Quorum sensing

The influence of *rpoS*, nutrient limitation and cell density on LuxS-mediated quorum sensing was determined according to the method of Bassler (Surette and Bassler, 1998). A *Vibrio harveyii* reporter strain (BB170) that is deficient in quorum sensing system 1 (homoserine lactone mediated) but capable of responding to quorum sensing system 2 autoinducers (LuxS-mediated) was used. *V. harveyi* BB170 was diluted from an overnight culture (18 hours after inoculation) to a density of 4×10^5 cfu / ml. *E. coli* supernatant (10 %) collected throughout the growth cycle was added to this culture and the resulting luminescence measured 2.5 hours later. At this time (2.5 hours after inoculation), the innate *V. harveyi* luminescence was at its minimum so any luminescence induced by *E. coli* supernatant could be measured. Specific luminescence was determined by dividing the light output by the light output from the positive control (light output from *V. harveyi* stationary phase supernatant).

Autoinducer 2 production (AI-2) (LuxS-mediated) was analyzed for high and low-density planktonic *E. coli* MC4100 and RH90. Production of LuxS autoinducer by *E. coli* varied significantly depending on *rpoS*, nutrient limitation and cell density. Overall, higher levels of AI-2 were detected in high density compared with low-density *E. coli* MC4100 and RH90. The general trend found was that AI-2 increased concurrent with entry into stationary phase and that elevated levels of AI-2 continued into stationary phase.

In high-density cultures there was a general trend of higher AI-2 levels in wild type (MC4100) compared with the *rpoS* null mutant strain (RH90). Overall, AI-2 was

found to increase upon entry into stationary phase in both MC4100 and RH90. However, the magnitude of the increase varied tremendously depending on the nutrient limitation. Considerably more AI-2 was found in magnesium- and phosphate-limited cells compared with carbon- and nitrogen-limited. In magnesium- and phosphate-limited MC4100 AI-2 levels were at least double that of RH90 (fig. 4-8, 4-9, 4-10, 4-11).

In low-density cells any increase in AI-2 occurred later compared with high-density cultures. Any increase in AI-2 occurred just after the onset of stationary phase in low-density cells, compared with 1 hour prior to entry into stationary phase in high-density cells. The overall levels of AI-2 were reduced in low-density compared with high-density cells (fig. 4-12, 4-13, 4-14, 4-15).

Figure 4-8. Effect of carbon limitation on LuxS-mediated quorum sensing determined by luminescence of *Vibrio harveyi* (bars) after the addition of *E. coli* RH90 (Δ *rpoS*) and MC4100 supernatant obtained throughout the growth cycle (filled circles) from cells grown to high-density in CDM_{10} at 37 °C with aeration.

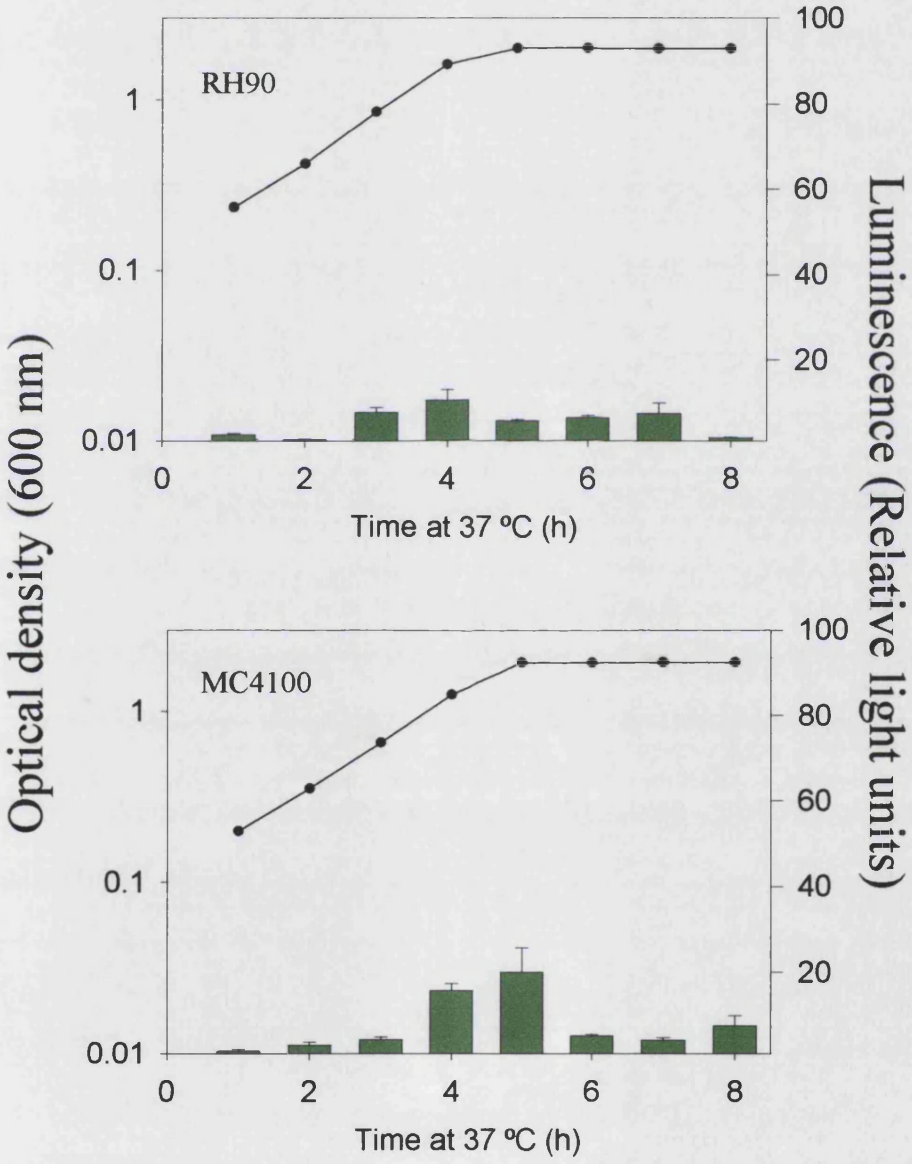


Figure 4-9. Effect of magnesium limitation on LuxS-mediated quorum sensing determined by luminescence of *Vibrio harveyi* (bars) after the addition of *E. coli* RH90 (Δ *rpoS*) and MC4100 supernatant obtained throughout the growth cycle (filled circles) from cells grown to high-density in CDM_{10} at 37 °C with aeration.

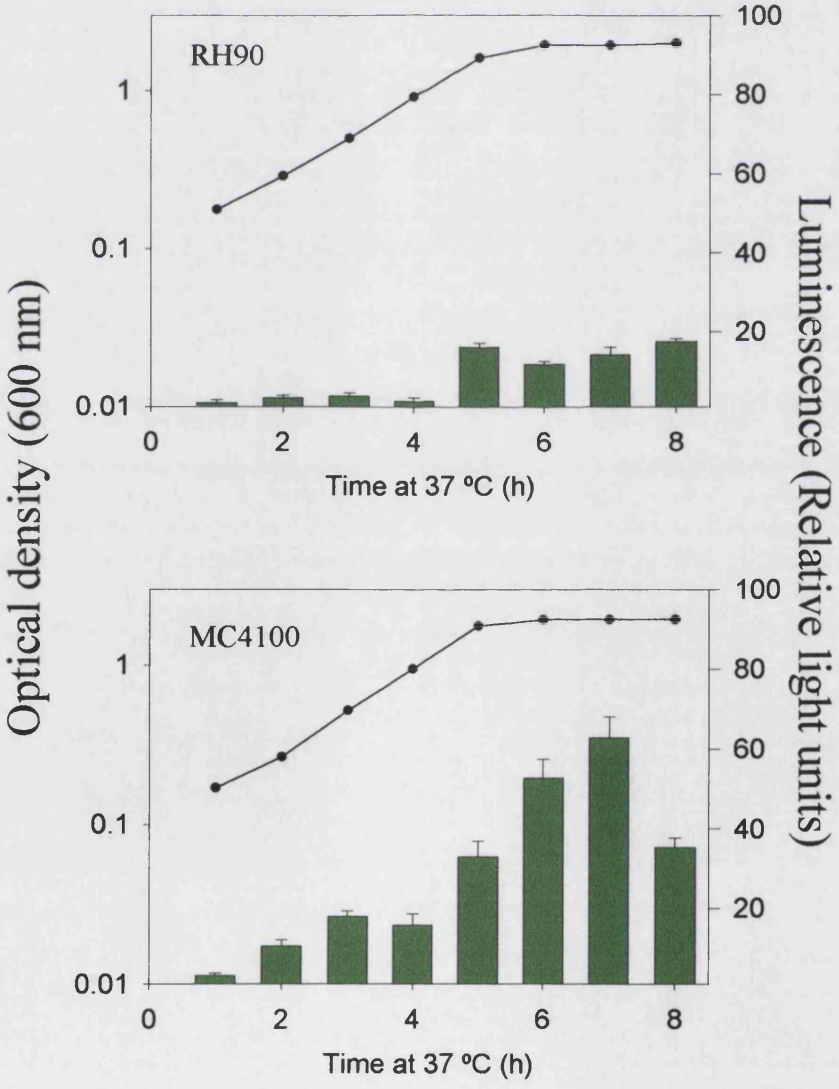


Figure 4-10. Effect of nitrogen limitation on LuxS-mediated quorum sensing determined by luminescence of *Vibrio harveyi* (bars) after the addition of *E. coli* RH90 (Δ *rpoS*) and MC4100 supernatant obtained throughout the growth cycle (filled circles) from cells grown to high-density in CDM_{10} at 37 °C with aeration.

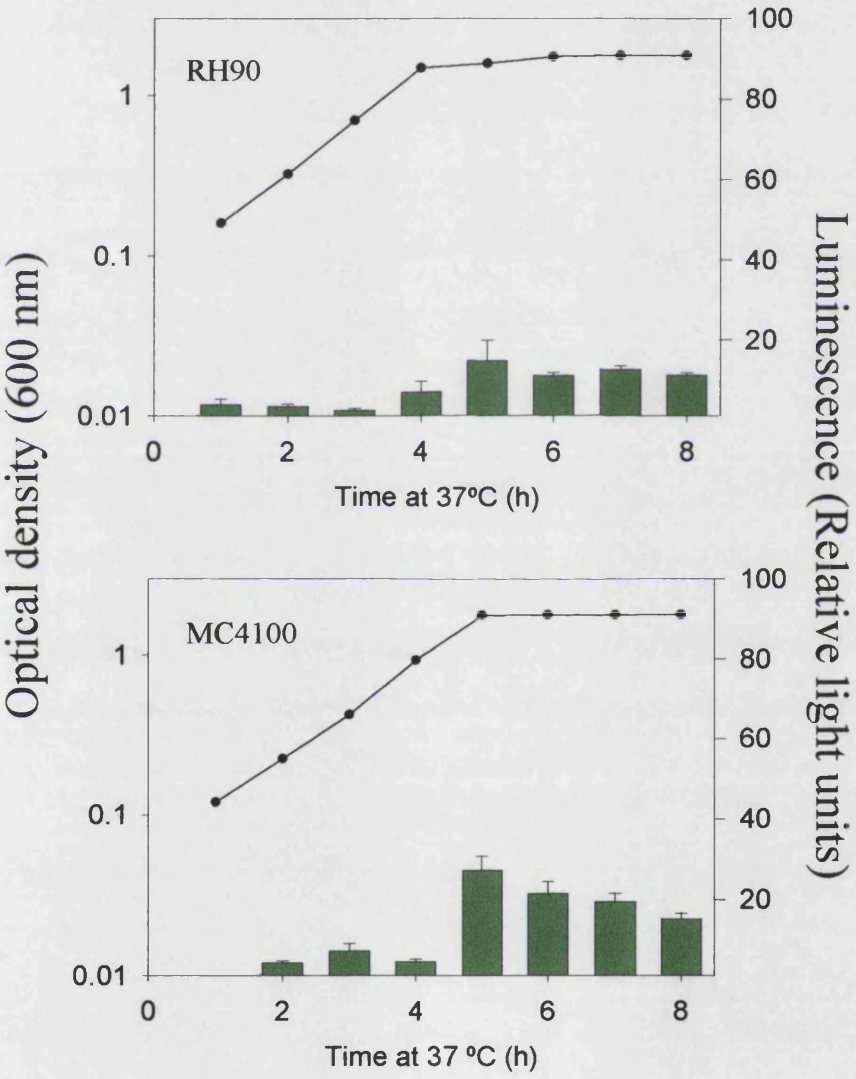


Figure 4-11. Effect of phosphate limitation on LuxS-mediated quorum sensing determined by luminescence of *Vibrio harveyi* (bars) after the addition of *E. coli* RH90 (Δ *rpoS*) and MC4100 supernatant obtained throughout the growth cycle (filled circles) from cells grown to high-density in CDM_{10} at 37 °C with aeration.

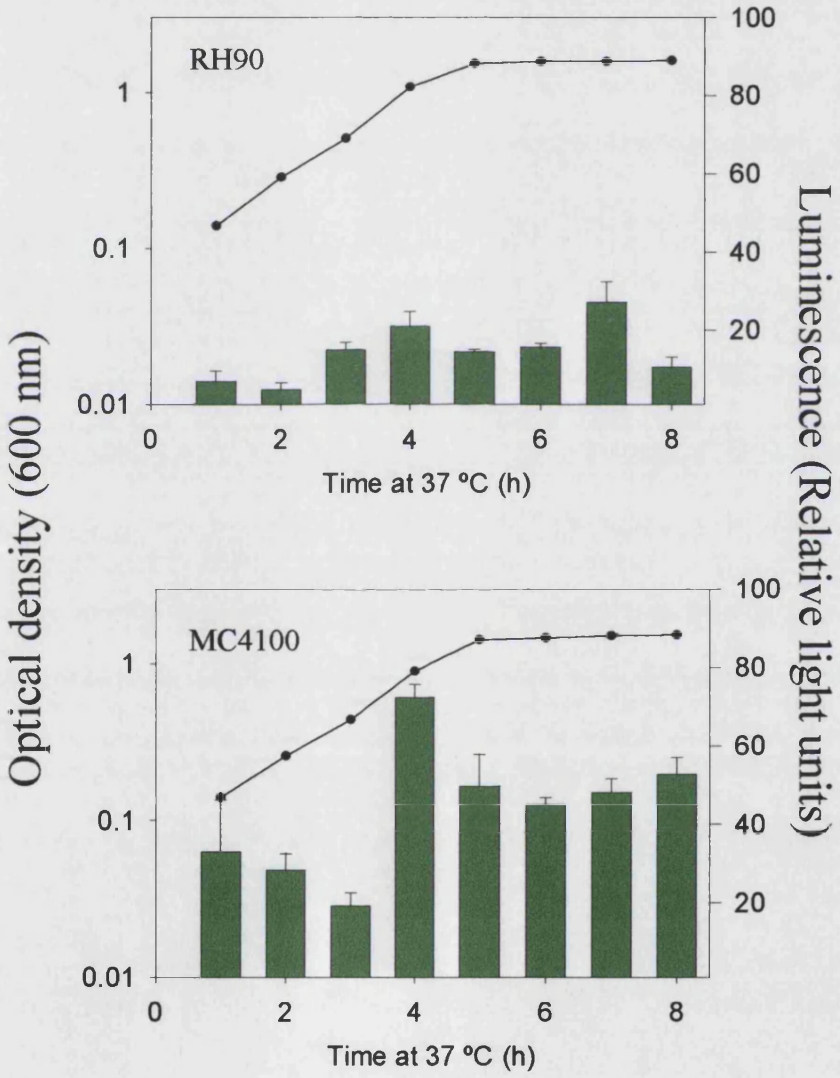


Figure 4-12. Effect of carbon-limitation on LuxS-mediated quorum sensing determined by luminescence of *Vibrio harveyi* (bars) after the addition of *E. coli* RH90 (Δ *rpoS*) and MC4100 supernatant obtained throughout the growth cycle (filled circles) from cells grown to low-density in CDM_{10} at 37 °C with aeration.

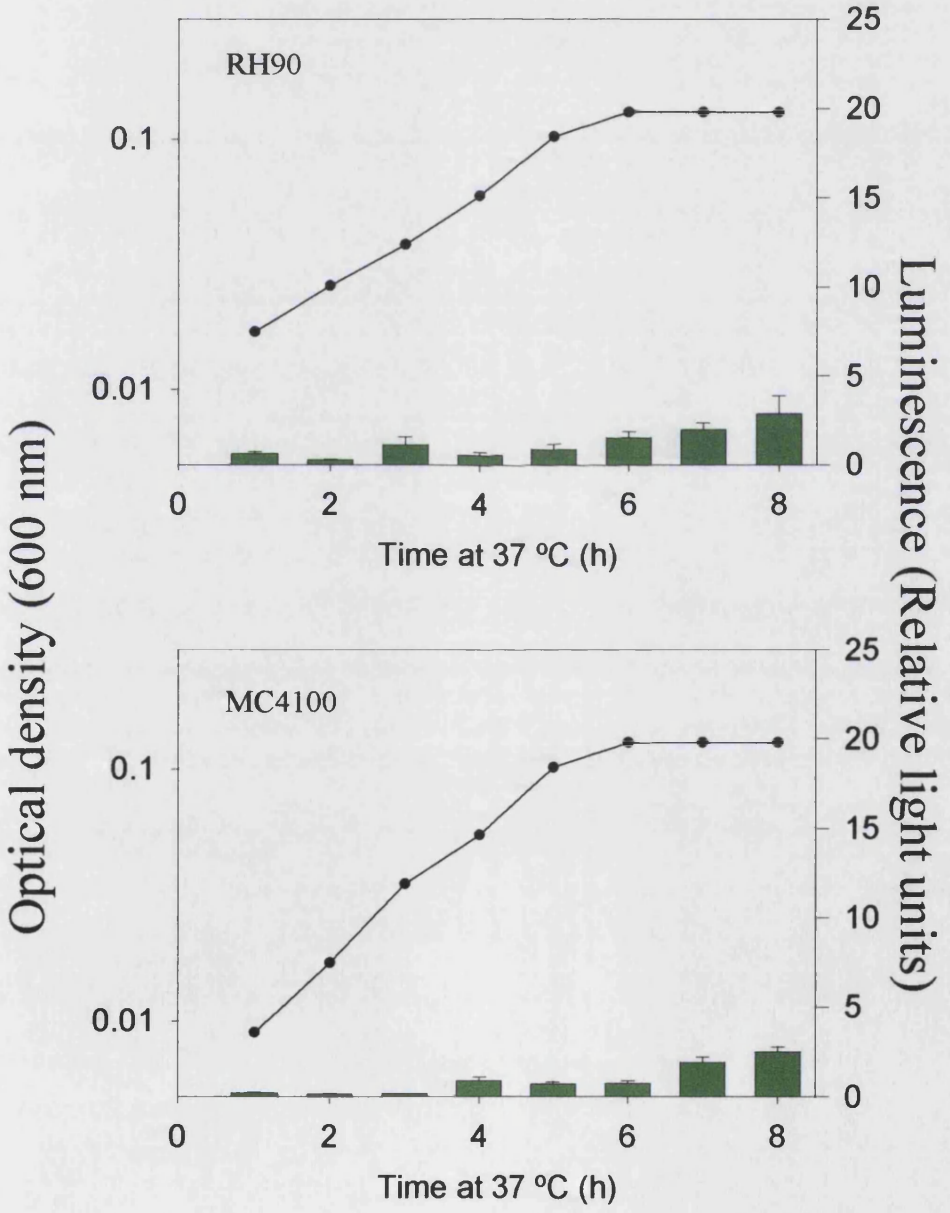


Figure 4-13. Effect of magnesium limitation on LuxS-mediated quorum sensing determined by luminescence of *Vibrio harveyi* (bars) after the addition of *E. coli* RH90 (Δ *rpoS*) and MC4100 supernatant obtained throughout the growth cycle (filled circles) from cells grown to low-density in CDM_{10} at 37 °C with aeration.

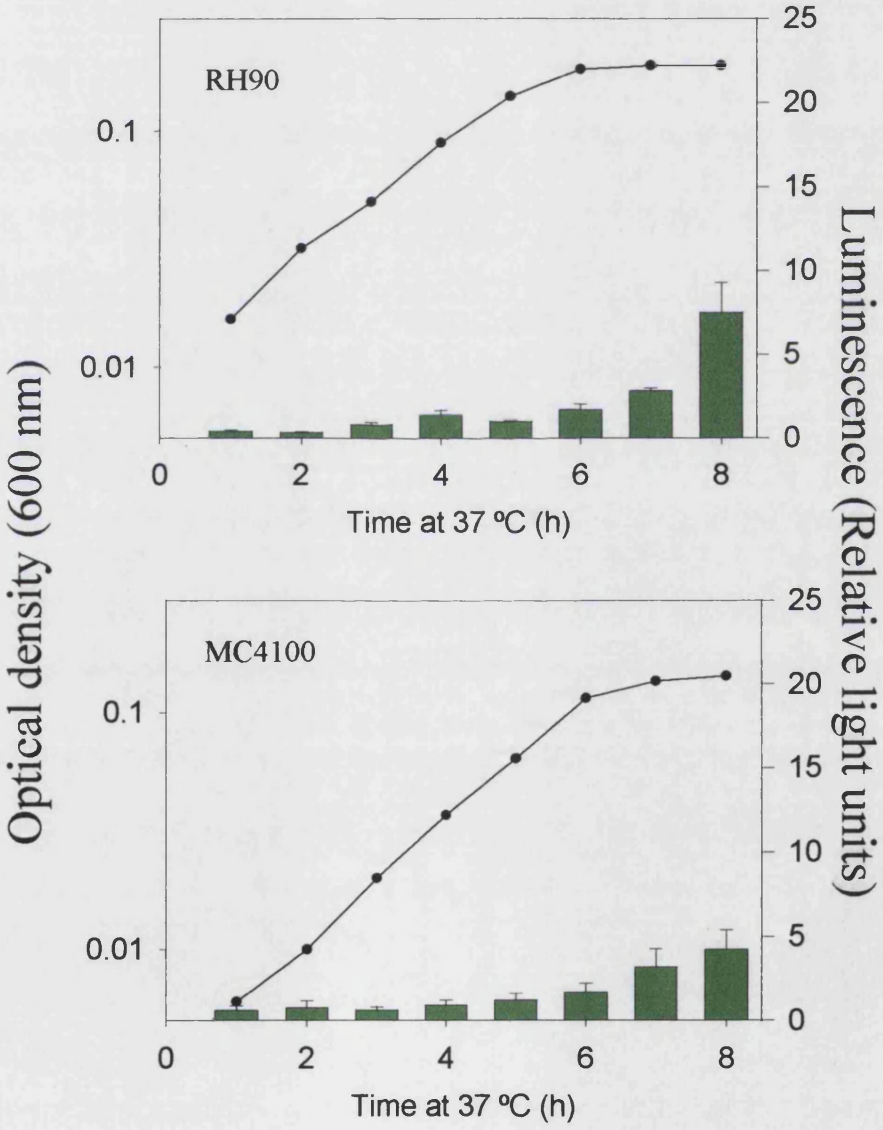


Figure 4-14. Effect of nitrogen limitation on LuxS-mediated quorum sensing determined by luminescence of *Vibrio harveyi* (bars) after the addition of *E. coli* RH90 (Δ *rpoS*) and MC4100 supernatant obtained throughout the growth cycle (filled circles) from cells grown to low-density in CDM_{10} at 37 °C with aeration.

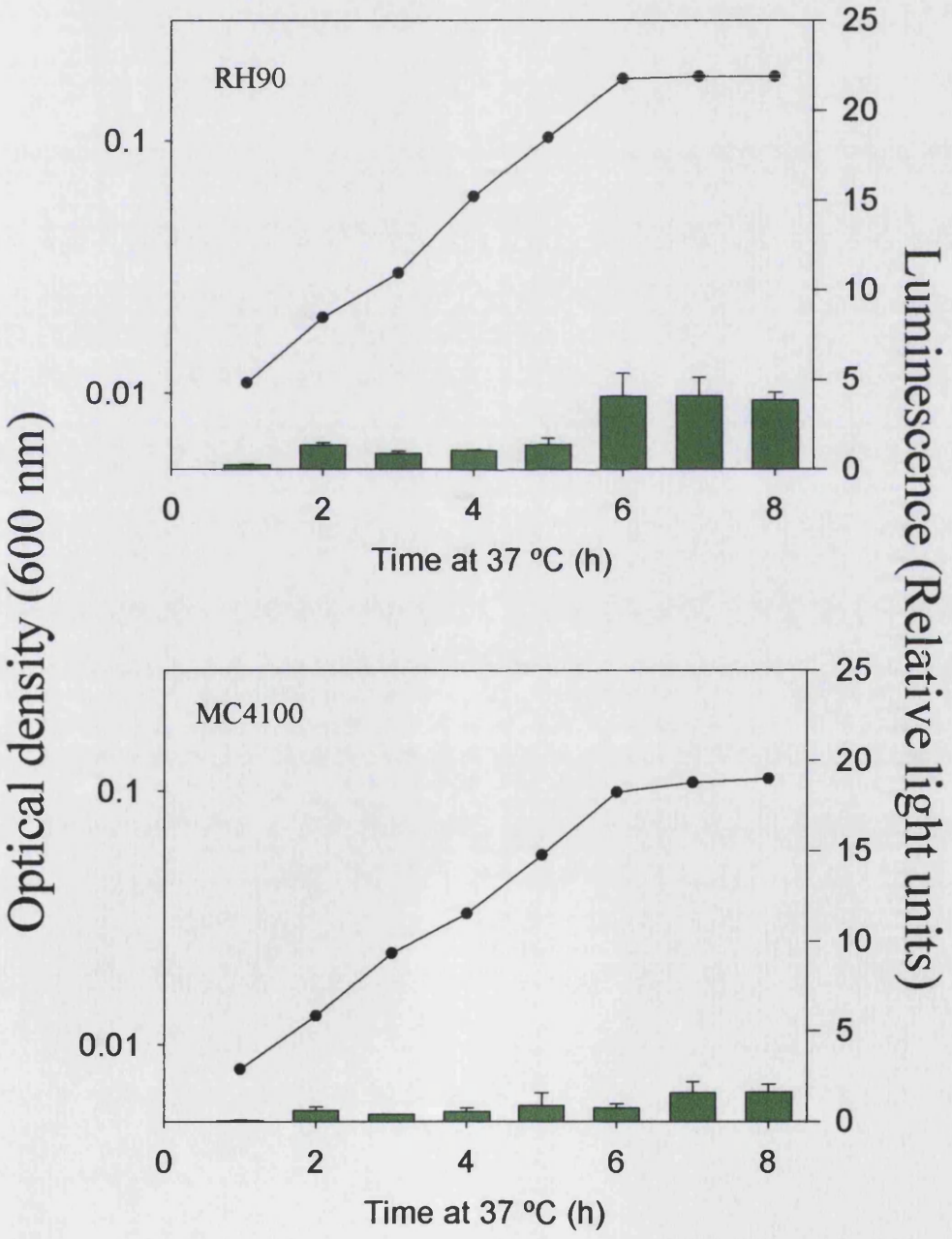
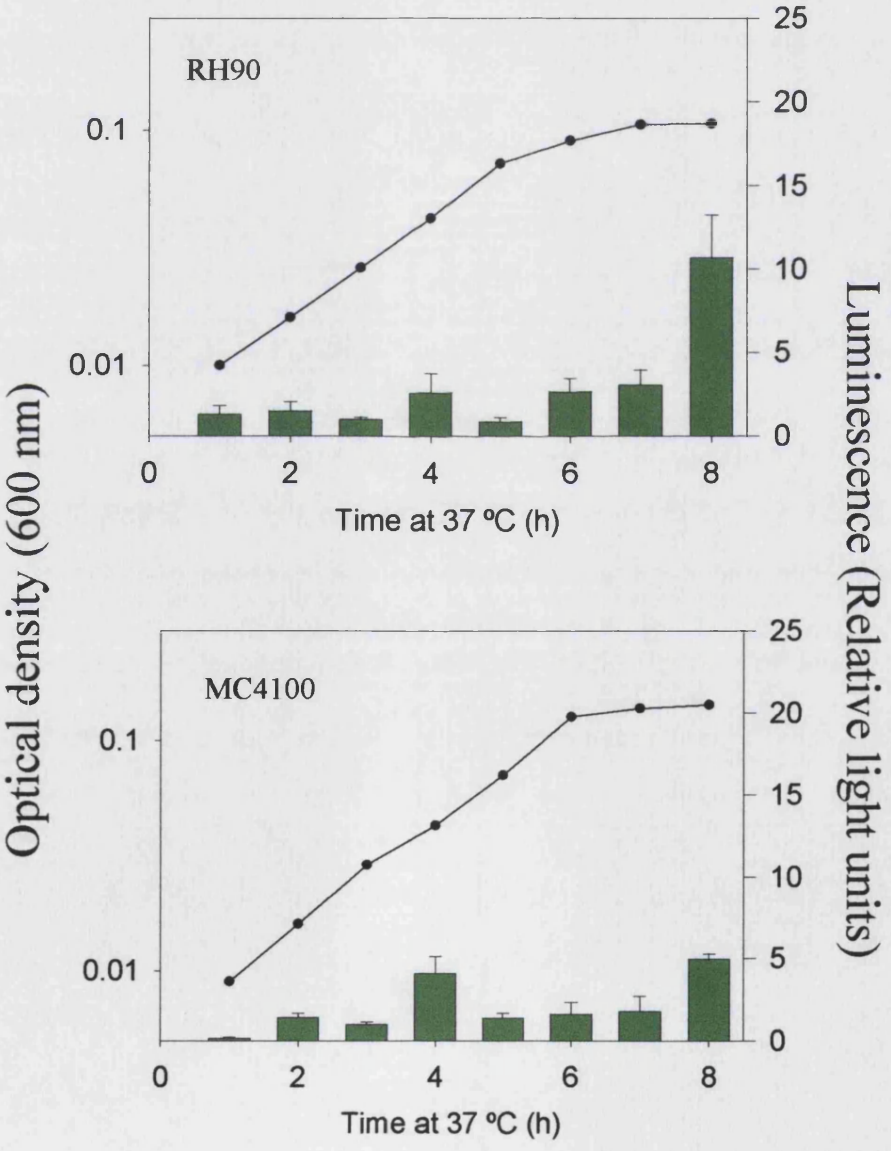


Figure 4-15. Effect of phosphate limitation on LuxS-mediated quorum sensing determined by luminescence of *Vibrio harveyi* (bars) after the addition of *E. coli* RH90 (Δ *rpoS*) (A) and MC4100 (B) supernatant obtained throughout the growth cycle (filled circles) from cells grown to low-density in CDM_{10} at 37 ° C with aeration.



4.3 DISCUSSION

4.3.1 Influence of *rpoS* and nutrient limitation on trehalose production

This study has examined the influence of *rpoS* and nutrient limitation on phenotypic changes associated with stationary phase. It was found that production of trehalose was strongly influenced by the specific nutrient limitation. Indeed, nutrient limitation had a greater effect on trehalose than did functional *rpoS*. Very little trehalose was detected under conditions of carbon limitation in planktonic culture: this result is not surprising, as a number of other studies have reported similar results (Germer et al., 1998; Notley and Ferenci, 1996). Although trehalose has been found in significant quantities in slow-growing, carbon-limited chemostat cultures (Liu et al., 2000; Notley and Ferenci, 1996) it has not been detected in carbon-limited batch culture (Germer et al., 1998; Notley and Ferenci, 1996). Previous studies have suggested that trehalose synthesis is inhibited under conditions of carbon limitation, as there is a reduction in the cellular content of the precursor UDP-glucose (Germer et al., 1998).

It is known that the operon responsible for trehalose synthesis, *otsA/B* is *rpoS* regulated (Hengge-Aronis et al., 1991). The highest levels of trehalose were found in magnesium- and phosphate-limited planktonic culture, concurrent with an increase in *rpoS* expression. As this study has shown that *rpoS* is not expressed in response to carbon and nitrogen limitation in planktonic culture (Chapter 3), it could be expected that no trehalose would be produced under these conditions or under any conditions in the *rpoS* null mutant RH90. However, trehalose was detected in RH90 (particularly in biofilm culture) and under conditions of nitrogen limitation. As σ^S does not have true promoter specificity and σ^{70} is capable of transcribing from *rpoS*-regulated promoters (Arnqvist et al., 1994) (Hengge-Aronis, 1999), it is feasible that in the

absence of *rpoS* other sigma factors (including σ^{54} under conditions of nitrogen limitation) may facilitate transcription of the *otsA/B* operon leading to trehalose production.

In planktonic culture no density-dependent differences in trehalose production were found. The effect of density on trehalose synthesis has not previously been studied in batch culture. However a chemostat study found that there was a ten-fold increase in trehalose at high population density but this was thought to result from a concurrent 8-fold increase in *rpoS* expression (Tweeddale et al., 1998).

The overall levels of trehalose were much higher when cells of both RH90 and MC4100 were grown as a biofilm: this may be indicative that a distinct 'biofilm phenotype' exists as suggested by other studies (Brown and Gilbert, 1993; Brown et al., 1988; Costerton, 1995). In particular, the level of trehalose in the *rpoS* null mutant (RH90) was elevated when cells were grown as a biofilm, suggesting that biofilm growth overrides any requirement for *rpoS* for trehalose production. It is also feasible that addition pathways (distinct from *otsA/B*) for trehalose production may exist and that these other pathways are utilized under conditions of biofilm growth or when *rpoS* is absent or not expressed. In Mycobacteria it is known that at least three separate pathways for trehalose biosynthesis exist (De Smet et al., 2000), it is possible that similar pathways may be present in *E. coli* and there are candidate genes with homology matches to mycobacterial *treY / treZ* in the *E. coli* genome.

4.3.2 The influence of *rpoS*, cell density and nutrient limitation on the fatty acid profile of *E. coli* MC4100 and RH90

This study has provided an analysis of the fatty acid profiles of nutrient-limited *E. coli*. CFA formation was not found to vary significantly with cell density. However, substantial variation in CFA content was found depending on the specific nutrient limitation. Carbon-limited planktonic cells had very low CFA levels, magnesium- and nitrogen-limited cells had intermediate levels and phosphate-limited cells had very high CFA content. This pattern is similar to that obtained with trehalose. Previous studies have analyzed CFA content in cells grown to stationary phase in rich media and have examined the expression of the CFA synthase gene rather than actual CFA levels (reviewed in (Grogan and Cronan, 1997)). To date, actual CFA levels have not been studied because of the technical difficulty in their isolation and because the reagents used to produce methyl esters of the fatty acids were thought to produce artifactual results (Grogan and Cronan, 1997). However, this study has used a reliable, highly reproducible method of methyl ester production that is based on alkaline hydrolysis and is not thought to produce artifactual results (Grogan and Cronan, 1997). It is preferable to study actual CFA levels rather than CFA synthase expression as its activity is only transient on entry into stationary phase (Chang et al., 2000), and so may not be detected and although CFA activity decreases, the process of CFA formation is irreversible (Chang et al., 2000).

In this study, CFA levels were generally lower in the *rpoS* null mutant (RH90) compared with the wild type strain (MC4100). Previous studies in rich media have found that CFA synthase activity is almost entirely abolished if *rpoS* is absent (Eichel et al., 1999). It is not surprising that CFA production was not abolished in RH90 in

this study as CFA synthase is known to have 2 promoters, one of which is σ^S-regulated and the other that is recognized by σ^{70} (Wang and Cronan, 1994). Previously, it has been suggested that the σ^{70}-regulated promoter is functional only in log phase and that the σ^S promoter takes over upon entry into stationary phase (Wang and Cronan, 1994). However, it is now known that the σ^{70} promoter can be activated in stationary phase and that in the absence of *rpoS*, σ^{70} can transcribe from both promoters (Eichel et al., 1999). As there is no strict consensus sequence for *rpoS* regulated promoters (Becker and Hengge-Aronis, 2001; Nguyen et al., 1993; Tanaka et al., 1995) it seems entirely feasible that in the absence of *rpoS*, σ^{70} (or other σ factors) can enable the transcription of CFA synthase.

It is interesting to note that the levels of CFA are higher in both RH90 and MC4100 carbon-limited biofilms. It could be expected that in MC4100, levels would be higher in biofilm culture as *rpoS* is expressed under these conditions. The observation that CFA content is higher even in the absence of *rpoS* suggests that as with trehalose, growth as a biofilm may override expression of 'normal' planktonic patterns of gene expression and enable development of a 'biofilm phenotype'.

Th analysis of total fatty acid profiles (displayed as dendrograms) does not provide specific information about the fatty acids related to stress responses and stationary phase. However, they are intriguing as they demonstrate the huge diversity in cell physiology that is achievable simply by imposing different nutrient limitations.

4.3.3 The influence of *rpoS*, cell density and nutrient limitation on LuxS-mediated quorum sensing

4.3.3.1 Influence of *rpoS* on quorum sensing in *E. coli*

LuxS production was strongly influenced by cell density and *rpoS*. The specific nutrient limitation had some effect on LuxS-mediated quorum sensing but this was not as striking as the influence of *rpoS* or cell density. High-density planktonic cells exhibited high-level autoinducer production if *rpoS* was functional (MC4100) and induced (under magnesium and phosphate limitation). If *rpoS* was absent (RH90) or not expressed (carbon and nitrogen limitation) large increases in LuxS-mediated quorum sensing were not seen. These results indicate that *rpoS* is involved in LuxS-mediated quorum sensing. These results are entirely novel, as the influence of *rpoS* on LuxS has not been studied previously. However, the influence of LuxS-mediated quorum sensing on *rpoS* has been studied using microarray analysis. AI-2 was found to have a weak stimulatory effect on *rpoS* at the transcriptional level (1.2 fold increase) but the effect on *rpoS* translation and protein stability has not been determined (DeLisa et al., 2001). The influence of *rpoS* has been established for AHL mediated quorum sensing in *P. aeruginosa*. RpoS was found to regulate AHL-mediated quorum sensing (Whiteley and Parsek, 2000). Recent studies have indicated that *rpoS* regulation of quorum sensing in *P. aeruginosa* may act via the stringent response and inorganic polyphosphate and it was suggested that the stringent response can activate AHL mediated quorum sensing independently of cell density (Van Delden et al., 2001). This situation seems unlikely to be mirrored in LuxS-mediated quorum sensing, as induction of *rpoS* could not induce quorum sensing independently of cell density. High-level LuxS production was not seen in planktonic cells limited to a low cell density in this study even if *rpoS* was expressed (in magnesium and

phosphate-limited cultures). It is also unlikely that *rpoS* regulation of AI-2 acts via the stringent response as exposure to serine hydroxymate (which causes the induction of ppGpp) had a negative effect on AI-2 production in a chemostat model (DeLisa et al., 2001).

4.3.3.2 Influence of growth phase on quorum sensing

Previously, it had been difficult to uncouple population density effects from growth rate effects in batch culture. In batch culture studies, it has been unclear whether patterns of LuxS autoinducer production are a result of population density or growth phase effects. This study has enabled the study of the separate influences of growth phase and density. Many previous studies have attributed AI-2 regulation to growth phase and have stated that AI-2 activity is maximal in 'mid-log' (Surette and Bassler, 1999; Surette et al., 1999), thus indicating that *rpoS* regulation of LuxS is unlikely. However, the use of the term 'mid-log' is dubious as the only fixed point on a growth curve (in batch culture) is stationary phase and 'mid-log' will vary depending on the inoculum. In fact, previous studies have found that AI-2 production is maximal around an OD (600nm) of 1.0 in complex media (Surette and Bassler, 1999; Sperandio et al., 1999). It is likely that *rpoS* regulated events begin around this point in complex media as growth begins to slow and stationary phase events begin. As LuxS-mediated quorum sensing seems to be regulated by *rpoS*, it is indirectly linked with growth phase, as *rpoS* is associated with entry into stationary phase. However, *rpoS* is not exclusively linked to stationary phase and may be induced in the log phase, particularly in response to osmotic stress (Hengge-Aronis, 1996). It would be interesting to determine if induction of *rpoS* in log phase leads to AI-2 production. However, even if *rpoS* was induced in log phase it seems likely that AI-2 production

would only be induced if the population was sufficiently dense (i.e. above the density of the low density cells used in this study).

4.3.3.3 Influence of cell density on quorum sensing.

In this study, it was found that in addition to *rpoS*, cell density strongly influences LuxS-mediated quorum sensing as high-level AI-2 production was not seen in low-density cells. This is not surprising as quorum sensing is by definition, a density-dependent phenomenon. It is likely that the low-density cells were limited at a population density below the 'quorum level'; that is, not sufficiently dense to initiate the phosphorylation cascade that results in light production. However, it is feasible that quorum sensing did occur in the low density cells and that the method of normalisation of data used in this study negates the true trend. Data were normalised with respect to the positive control (*V. harveyi* stationary phase supernatant). This method does not allow for the influence of cell density on LuxS production, that is, supernatant from low-density cells will intrinsically have less autoinducer activity than that obtained from high-density cells, as fewer cells were present to produce autoinducer molecules. If this study were repeated, it would be useful to measure the range over which there is a linear relationship between cell density and luminescence, to use only supernatant from cells within this range and normalise the data for the original cell number.

4.3.3.4 Influence of nutrient limitation on quorum sensing

The influence of nutrient limitation on AI-2 production was also studied. It is known that nutrient limitation has an effect on AI-2 production as increased levels were found under conditions of magnesium and phosphate limitation even if *rpoS* was

absent (RH90). The increased AI-2 production under these conditions is not due to any component of the CDM_{10} because when fresh CDM_{10} (10 %) was added to *V. harveyi* it had no effect on AI-2 levels. However, the magnitude of the effect of nutrient limitation is only small compared with that of *rpoS* and cell density. Previous studies have suggested that σ^{54} may be involved in AI-2 mediated quorum sensing (DeLisa et al., 2001; Bassler et al., 1994). If σ^{54} had an important role in AI-2 mediated quorum sensing it could be expected that conditions of nitrogen limitation (where σ^{54} could be induced) might lead to high-level AI-2 production. This study did not find high-level AI-2 production under conditions of nitrogen limitation. Therefore, it would be interesting to determine if σ^{54} is induced in response to the conditions of nitrogen limitation used in this study.

Many of the phenotypic changes studied in this chapter are associated with increased resistance to stress. Therefore, it was interesting to determine the influence of nutrient limitation and *rpoS* on the resistance of *E. coli* to stress and ascertain if there is any correlation between the phenotypic changes examined in this chapter and resistance to stress. This will be examined in the following chapter.

5 INFLUENCE OF *RPOS* AND NUTRIENT LIMITATION ON SUSCEPTIBILITY OF *E. COLI* TO STRESS

5.1 INTRODUCTION

Bacteria in the domestic environment are frequently exposed to stress including that of biocides, desiccation, heat shock and nutrient limitation.

5.1.1 Heat shock response

Exposure to temperatures in excess of 40 °C causes the rapid induction of at least 20 heat shock proteins (HSPs). HSPs are typically molecular chaperones such as the DnaK-DnaJ-GrpE complex or ATP dependent proteases such as Lon and Clp (reviewed in Arsene et al., 2000;Yuru et al., 2000). HSPs are important for cell survival in response to high temperature as they prevent protein aggregation and play a major role in protein folding, repair and degradation. HSPs may also be induced in response to other stresses such as carbon or amino acid starvation, exposure to DNA damaging agents, antibiotics, heavy metals, oxidative stress or phage infection. However, the mechanism of induction and protection afforded under these conditions is not well understood (Yuru et al., 2000).

The induction of HSPs in response to high temperature is controlled by two alternative sigma factors, σ^{32} (encoded by *rpoH*) and σ^{E} (Yuru et al., 2000). The heat shock associated sigma factor σ^{32} protects against cytoplasmic damage and is thought to act as a cellular thermosensor. At ambient temperature *rpoH* mRNA forms a complex secondary structure that inhibits translation. However, in response to elevated temperature the secondary structure melts and translation is initiated (Yuru et

al., 2000). Induction of σ^{32} directs RNA polymerase holoenzyme towards the transcription of HSPs. The other heat shock-associated sigma factor σ^{E} is induced in response to extreme heat shock (in excess of 50 °C) and protects against extracytoplasmic damage (Yuru et al., 2000). σ^{E} is responsible for the production of periplasmic proteases, chaperones and outer membrane proteins such as SurA and Skp (Arié et al., 2001; Rouviere and Gross, 1996; Schäfer et al., 1999). Interestingly, this sigma factor is also induced in response to ethanol exposure and misfolded membrane or periplasmic proteins (Yuru et al., 2000).

In addition to the specific heat shock response, the *rpoS* regulated general stress response is thought to contribute to survival during exposure to high temperature (Hengge-Aronis, 2000). The disaccharide trehalose plays a minor role in thermotolerance (Lange and Hengge-Aronis, 1991; McCann et al., 1991). The partially σ^{S}-dependent *htrE* operon (encoding proteins involved in the post-secretional assembly of pilus proteins) has also been implicated in thermotolerance (Raina and Georgopoulos, 1991). However, *otsAB* or *htrE* null mutations confer much weaker phenotypes than *rpoS* null mutations. Therefore, the genes identified to date cannot account fully for σ^{S}-dependent stationary phase thermotolerance (Hengge-Aronis, 2000).

5.1.2 Resistance to desiccation

A common stress encountered by bacteria in the natural environment is that of desiccation. Desiccation is particularly damaging as it causes changes in the physical state of membrane lipids and alters the structure of sensitive proteins (Leslie et al.,

1995). In addition to its role in thermotolerance and osmoprotection, trehalose plays a major role in resistance to desiccation (Welsh and Herbert, 1999; Leslie et al., 1995).

5.1.3 Triclosan

Triclosan [5-chloro-2 (2,4-dichlorophenoxy) phenol] is one of the most widely used biocides and is incorporated into fabrics, plastics and a variety of personal care products such as handsoaps, toothpastes and lotions (Bhargava and Leonard, 1996). In addition to its antibacterial effect, it is thought to possess anti-inflammatory properties (Barkvoll and Rolla, 1995). Triclosan is a broad-spectrum antimicrobial agent active against a wide variety of Gram-positive and Gram-negative bacteria, as well as fungi and yeast (Bhargava and Leonard, 1996). It was initially thought that the activity of triclosan resulted from non-specific disruption of the cell membrane (McDonnell and Russell, 1999), but it is now known that it results from the inhibition of a specific intracellular target. The target of triclosan in *E. coli* is the FabI (EnvM) class of enoyl-acyl carrier protein reductase (ENR) that are involved in bacterial fatty acid synthesis (McMurry et al., 1998; Heath et al., 1999). Therefore, unlike many biocides it does not affect multiple targets. This may explain why plasmid-encoded triclosan-resistant mutants have been isolated (McMurry et al., 1998).

5.1.4 Survival during long term starvation

In most natural environments, bacteria spend the majority of their existence under conditions of starvation (Finkel et al., 2000). Therefore, studies of bacteria undergoing long-term starvation can provide insights into survival mechanisms. Studies of the long-term survival of bacteria grown in batch culture date back to the early half of the 20th century (Steinhaus and Birkeland, 1939). It was found that

cultures incubated in nutrient broth for 2 years (without the addition of nutrients) retained significant numbers of viable cells (Steinhaus and Birkeland, 1939). Recently, studies have focused on how novel mutations confer a competitive advantage during long-term starvation. These cells from aged stationary phase cultures exhibited what is now called the growth advantage during stationary phase, or GASP, phenotype (Zambrano et al., 1993; Zambrano and Kolter, 1993). A number of beneficial mutations have been identified which confer a GASP phenotype. Interestingly, the first GASP-conferring mutation to be identified was for partial loss of function of RpoS (Zambrano et al., 1993). Since then additional mutations have been identified which also confer a GASP phenotype (Zinser and Kolter , 2000; Zinser and Kolter, 1999). These mutations are concerned with amino acid catabolism and it has been suggested that GASP mutations allow enhanced catabolism of cellular components, thus enabling the cells to scavenge amino acids released from dead cells and continue growth and cell maintenance during starvation conditions (Finkel et al., 2000).

5.2 RESULTS

5.2.1 Effect of *rpoS* and nutrient limitation on susceptibility to heat at 52°C

The susceptibility of stationary phase (6 hours after entry) nutrient-limited planktonic and biofilm *E. coli* MC4100 and RH90 (Δ *rpoS*) to heat at 52 °C was determined. To ascertain if cell density influenced the susceptibility of *E. coli* to heat, planktonic cultures were tested after growth to high- and low-density. When carbon-limited cultures were examined it was found that overall, low-density planktonic culture was more resistant to exposure to heat at 52 °C than the corresponding high-density culture. The absence of *rpoS* (RH90) was found to have no effect on the

susceptibility of high-density planktonic culture to heat. However, when low-density planktonic culture was examined *rpoS* was found to influence the susceptibility of *E. coli* to heat as the *rpoS* null mutant (RH90) was more sensitive than the corresponding wild type (MC4100) (fig. 5-1). Biofilm cells were considerably (in excess of 2 log cycles) more resistant than either the high- or low-density planktonic culture (fig. 5-2). Interestingly, *rpoS* had no effect on the sensitivity to heat in biofilm carbon-limited cells.

When magnesium-limited culture was examined it was found that high–density planktonic culture was more resistant to heat at 52 °C than the corresponding low-density culture. *RpoS* was found to influence the susceptibility to heat but this effect was far more pronounced in the low- compared with the high-density culture (fig. 5-3). Magnesium-limited biofilms were more resistant than the low density planktonic culture and in the absence of *rpoS* (RH90) biofilms were more susceptible to heat at 52 °C (fig. 5-4).

Nitrogen-limited planktonic cells was exposed to heat at 52 °C and it was found that high-density culture was considerably more resistant than low-density culture. RpoS effected susceptibility to heat only in low-density planktonic culture; wild type cells (MC4100) were more resistant than RH90 (Δ *rpoS*) (fig. 5-5). When nitrogen-limited biofilm cultures were examined they were found to more resistant than the corresponding low density planktonic cells but not high-density planktonic cells (fig. 5-6).

The susceptibility of phosphate-limited planktonic cells to heat at 52 °C was strongly effected by *rpoS*, particularly in low-density cells. Cells lacking *rpoS* (RH90) showed considerably less resistance to heat. Overall, high-density planktonic cells were more resistant than the corresponding low-density cells (fig. 5-7). Biofim cells also exhibited *rpoS*-dependent heat resistance. Biofilms lacking *rpoS* (RH90) were considerably more sensitive to heat at 52 °C than the corresponding wild type cells (MC4100). In addition, biofilms were more resistant than the corresponding low-density planktonic culture (fig. 5-8).

Therefore, the general trend found was that high-density cells were more resistant than the corresponding low-density culture and that any *rpoS* effect was more pronounced in low- compared with high-density culture. In addition, biofilm cells were generally more resistant than the corresponding low-density planktonic culture but not the high-density planktonic culture.

Bar charts were constructed to illustrate the surviving fraction present after 50-min exposure to heat at 52 °C (fig. 5-9). In high-density planktonic culture the one striking result is that carbon-limited cells are extremely susceptible to heat at 52 °C and the surviving fraction was below the limit of detection. If high-density wild type cells are considered, overall there is no significant difference between magnesium-, nitrogen- and phosphate-limited cultures in terms of susceptibility to heat as all show between 0.08 and 0.12 surviving fraction after 50 min. The *rpoS* null mutant cultures (RH90) are generally more susceptible than their wild type counterparts. Nitrogen- and magnesium-limited RH90 show similar levels of susceptibility to heat (with 0.04 and 0.06 surviving fraction after 50 min, respectively). Phosphate-limited RH90 (Δ

rpoS) were very susceptible to heat and only 0.004 surviving fraction after 50 min of exposure to 52 ° C (fig. 5-9).

There are density-dependent effects in terms of sensitivity of *E. coli* to heat as low-density planktonic cells show a totally different pattern of sensitivity from the corresponding high-density cells. Generally, low-density planktonic cells are less resistant to exposure to heat at 52 ° C than the corresponding high-density cells. Interestingly, phosphate-limited low-density cells were the most susceptible to heat at 52 ° C, only 0.00007 surviving fraction of the wild type (MC4100) remained and the *rpoS* null mutant was below the level of detection after 50 min exposure to heat at 52° C. Additionally, the presence of *rpoS* had a strong effect on susceptibility to heat for low-density planktonic carbon- and nitrogen-limited cultures. However, magnesium-limited cells did not exhibit the same effect; in fact the *rpoS* null mutant (RH90) appeared to be slightly more resistant than the wild type (fig. 5-9).

Overall, it was found that biofilm culture was more resistant than the corresponding low-density planktonic culture but showed similar resistance to high-density planktonic culture. When cells are grown as a biofilm, the specific nutrient limitation appears to have little influence on the susceptibility to heat as the surviving fraction after 50 min varies only between 0.003 and 0.04 surviving fraction under all nutrient limitations tested. Interestingly, the susceptibility of nitrogen-limited cultures was only marginally influenced by the culture conditions as the surviving fraction after 50 min was between 0.003 and 0.04 surviving fraction under all culture conditions tested. Under conditions of biofilm growth, the effect of *rpoS* on susceptibility to heat was not apparent. Under some nutrient limitations the *rpoS* null mutant (RH90) was more

resistant than the wild type (MC4100) (phosphate limitation) whereas under other conditions the wild type showed increased resistance compared with the null mutant (magnesium and nitrogen limitation) (fig. 5-9).

Figure 5-1. Influence of carbon limitation on susceptibility to heat at 52 °C of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} to high- and low –density at 37 °C with aeration. (BLD indicates below the level of detection).

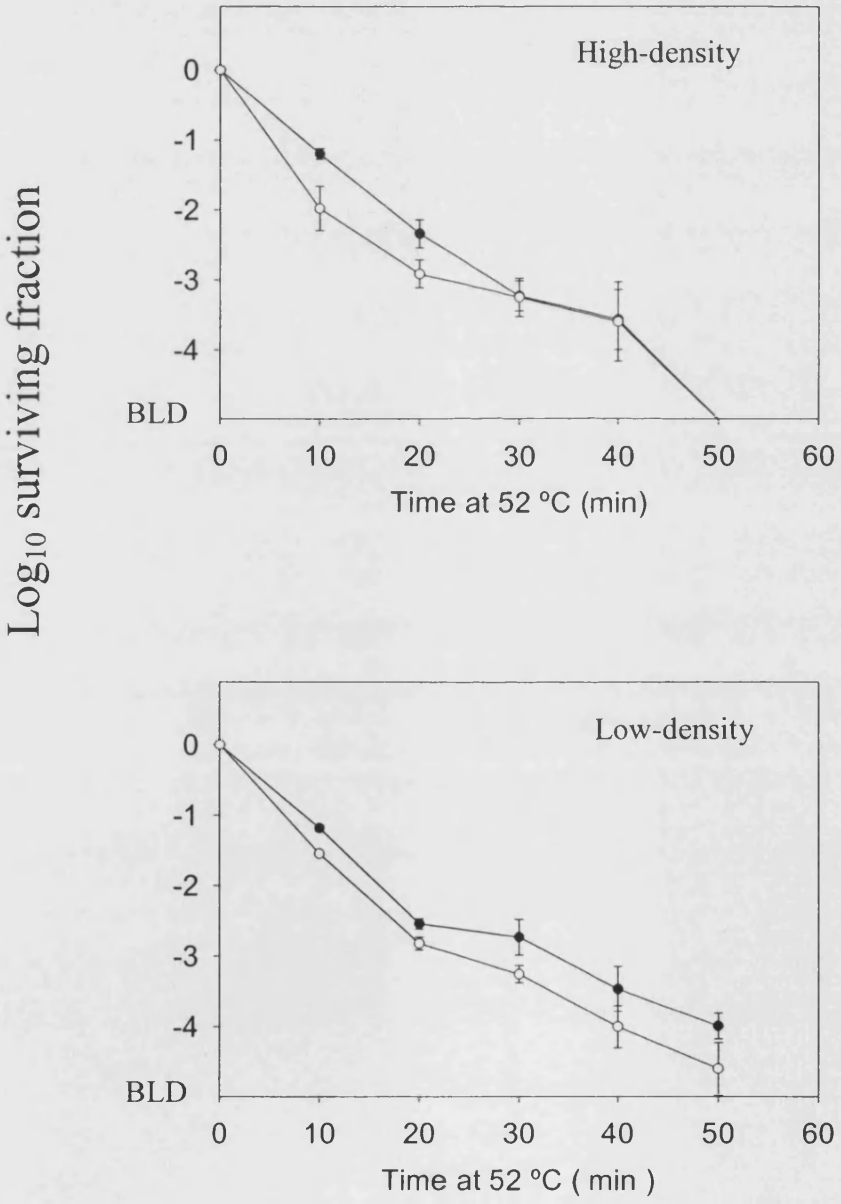


Figure 5-2. Influence of carbon limitation on susceptibility to heat at 52 °C of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} at 37 °C.

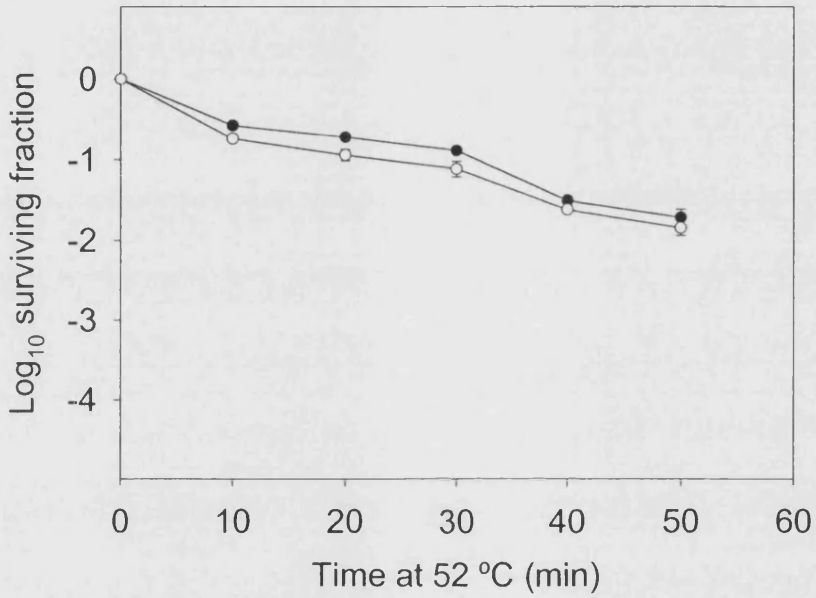


Figure 5-3. Influence of magnesium limitation on susceptibility to heat at 52 °C of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} to high- and low-density at 37 °C with aeration.

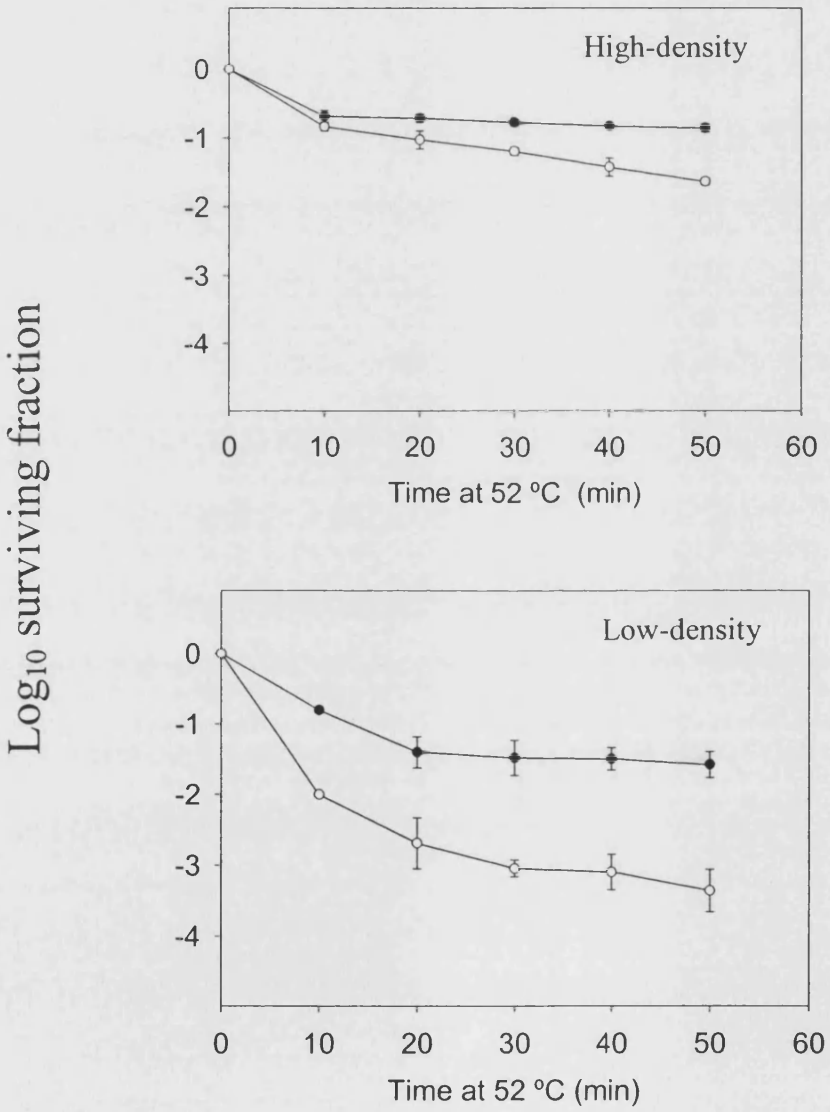


Figure 5-4. Influence of magnesium limitation on susceptibility to heat at 52 °C of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} at 37 °C.

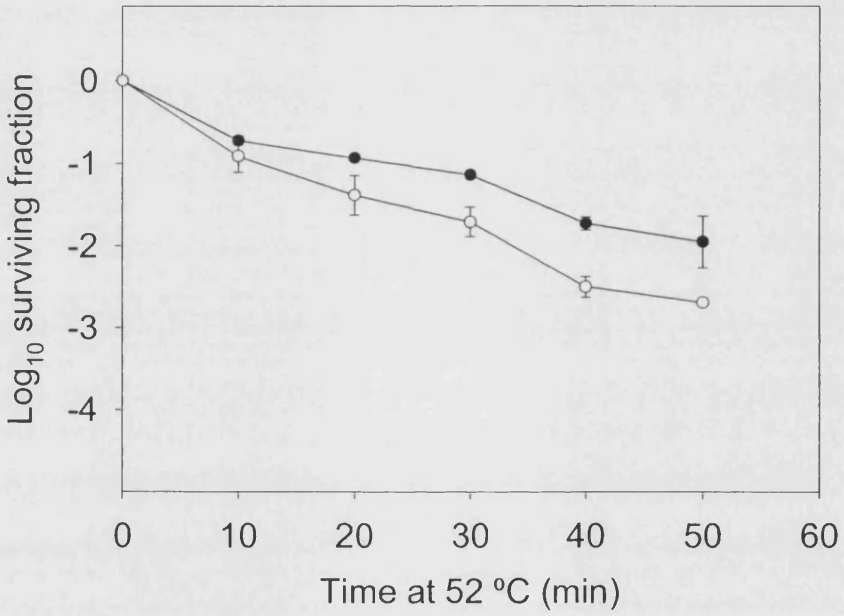


Figure 5-5. Influence of nitrogen limitation on susceptibility to heat at 52 °C of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} to high- and low-density at 37 °C with aeration.

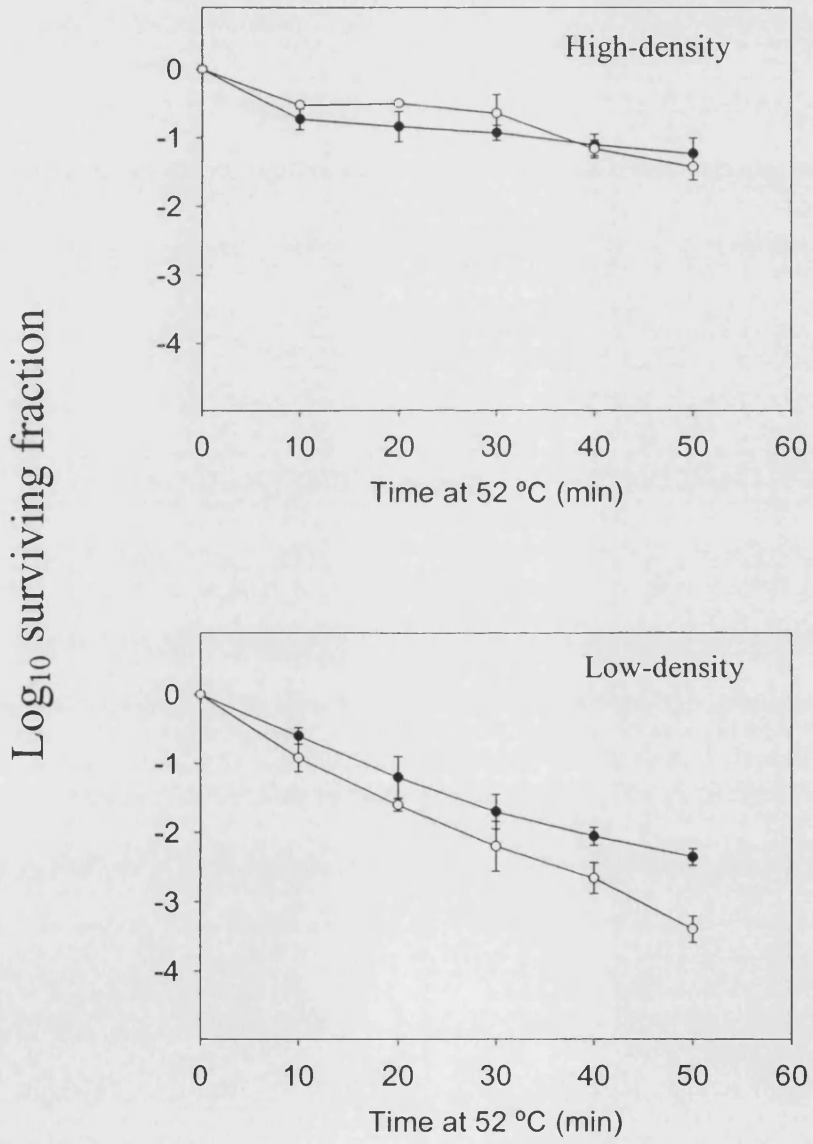


Figure 5-6. Influence of nitrogen limitation on susceptibility to heat at 52 °C of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} at 37 °C.

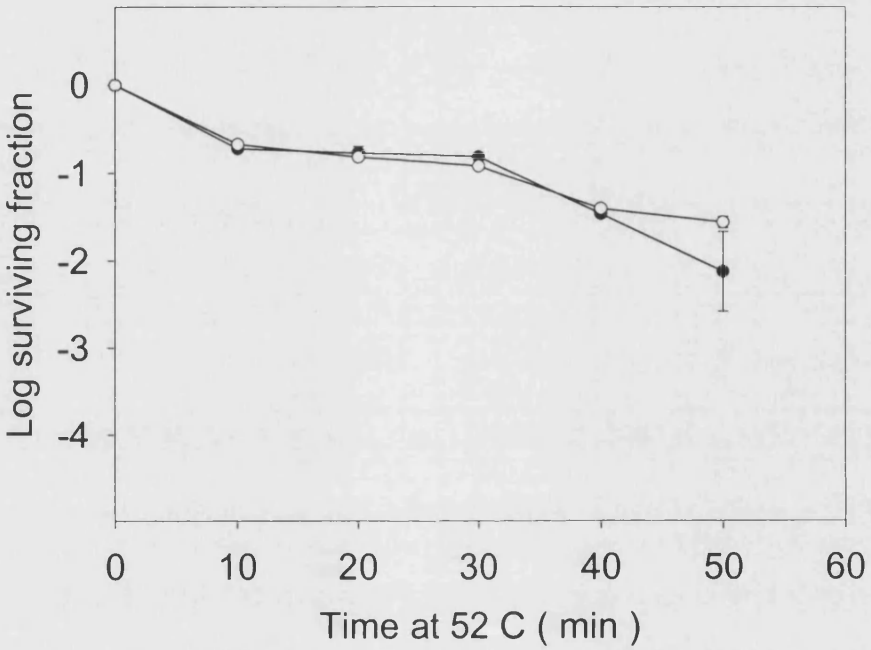


Figure 5-7. Influence of phosphate limitation on susceptibility to heat at 52 °C of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} to high- and low-density at 37 °C with aeration.

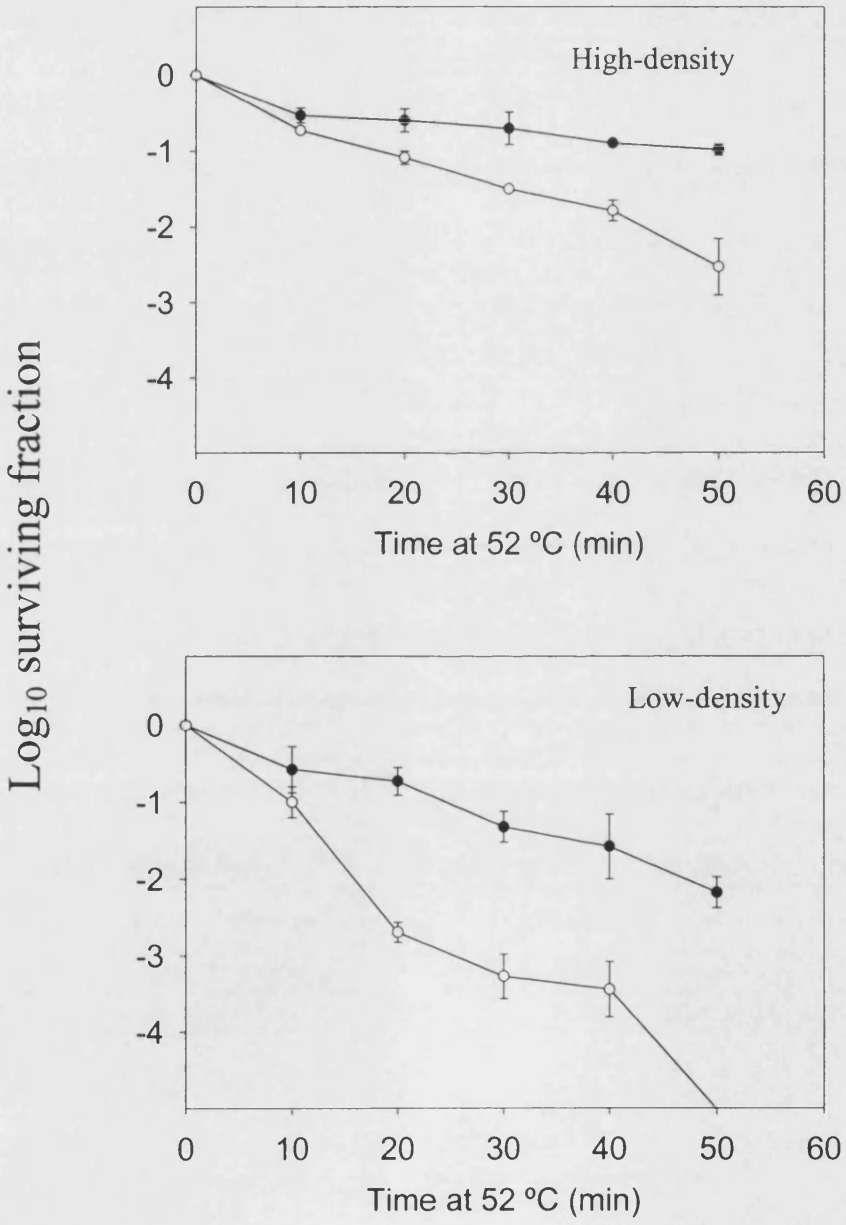


Figure 5-8. Influence of phosphate limitation on susceptibility to heat at 52 °C of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} at 37 °C.

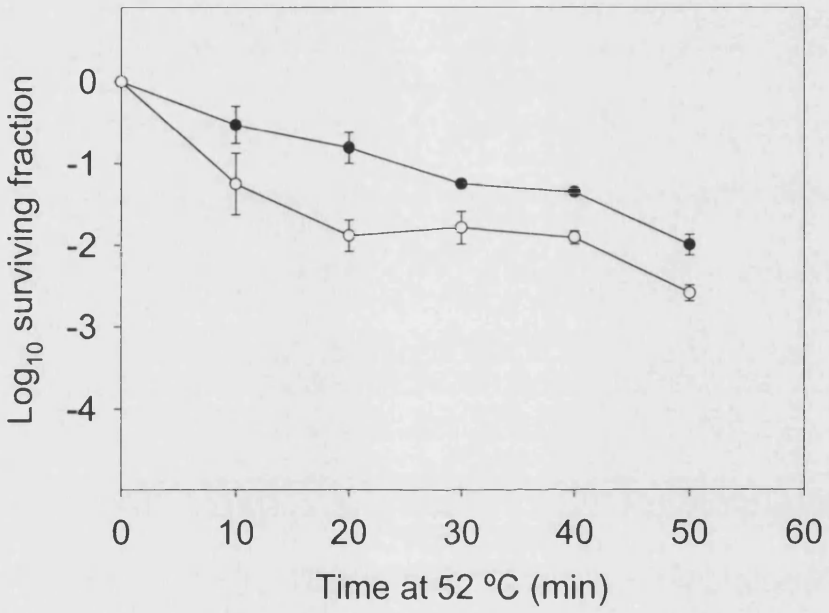
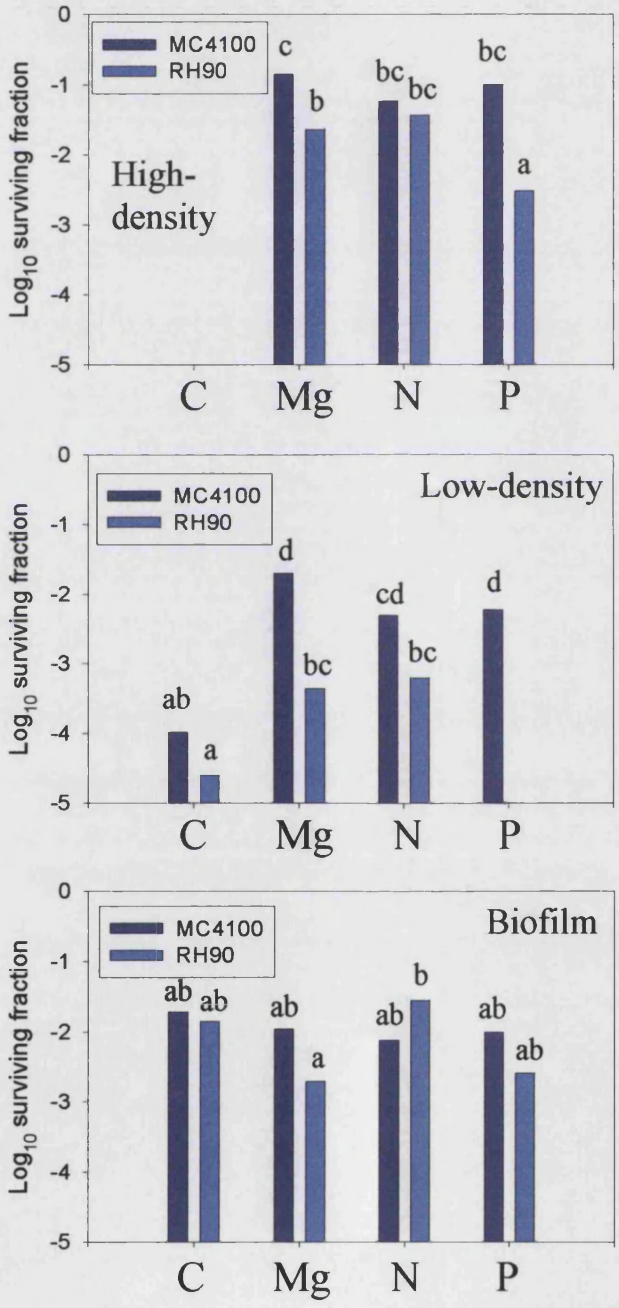


Figure 5-9. Effect of *rpoS* and nutrient limitation on susceptibility to heat at 52 °C for 50 min of stationary phase (6 hours after entry) high- and low-density planktonic and biofilm *E. coli* MC4100 and RH90.



5.2.2 Effect of *rpoS* and nutrient limitation on susceptibility to 15 μg / ml triclosan

The susceptibility to 15 μg /ml of triclosan of stationary phase (6 hours after entry) nutrient-limited planktonic and biofilm *E. coli* MC4100 and RH90 (Δ *rpoS*) was determined. To ascertain if cell density influenced the susceptibility of *E. coli* to heat, planktonic cultures were tested at both high- and low-density. When carbon-limited cultures were examined it was found that overall, low-density planktonic culture was marginally more resistant to triclosan than the corresponding high-density culture. The absence of *rpoS* (RH90) was found to have no effect on the susceptibility of planktonic carbon-limited *E. coli* to triclosan. However, the wild type was more resistant (almost a log cycle) than the *rpoS* null mutant if cells were cultured as a biofilm. Biofilm cells were substantially more resistant than either the high- or low-density planktonic culture (fig. 5-10, 5-11).

Magnesium-limited cells exhibited some density-dependent effects on susceptibility to triclosan: high-density cells were overall, more resistant than low-density cells. The susceptibility of magnesium-limited cells to triclosan was massively influenced by *rpoS*. Wild type cells were approximately 2 log cycles more resistant to triclosan than the corresponding *rpoS* null mutant cells if cells were cultured planktonically. Biofilm cells also exhibited a large *rpoS*-dependent effect. Unlike carbon-limited cells, magnesium-limited biofilms were not vastly more resistant than their planktonic counterparts. The wild type magnesium-limited biofilm cells were in fact, slightly less resistant than the high-density planktonic cells (fig. 5-12, 5-13).

When cells were nitrogen-limited, *rpoS* had only a negligible influence on susceptibility to triclosan. No difference was seen between wild type (MC4100) and *rpoS* null mutant (RH90) strains in high-density culture and there was a negligible, (less than one log cycle) difference in low-density cells. Similarly, no *rpoS* effect was seen in nitrogen-limited biofilm cells. Overall, nitrogen-limited cells were extremely resistant to exposure to triclosan (fig. 5-14, 5-15).

When phosphate-limited cells were exposed to triclosan, *rpoS* had a dramatic effect on their susceptibility. Wild type cells were more resistant to triclosan than *rpoS* null mutant cells (RH90) in both planktonic and biofilm culture. The magnitude of the *rpoS* effect was most pronounced in low-density planktonic culture where there was approximately a 3 log cycle difference between MC4100 and RH90. Some density-dependent effects on susceptibility to triclosan were seen in planktonic culture; however, the trend was complex. Wild type cells (MC4100) were more resistant if cultured to low cell density whereas the *rpoS* null mutant (RH90) was more resistant if grown to high-density (fig. 5-16, 5-17).

Bar charts were constructed to illustrate the surviving fraction present after 50 min exposure to triclosan (fig. 5-18). Presenting the data in this way made a number of trends discernable. In planktonic culture a strong *rpoS*-dependent effect was exhibited only by magnesium- and phosphate-limited cells and was not seen in carbon- and nitrogen-limited cells. Carbon-limited cells were highly sensitive to triclosan and no surviving cells were detected after 50 min exposure. Phosphate- and magnesium-limited cells were of intermediate resistance and nitrogen-limited cells were highly resistant to exposure to triclosan (with 0.07 to 0.09 surviving fraction

remaining after 50 min). Biofilm-cultured cells showed a completely different pattern of resistance to their planktonic counterparts. Nitrogen-, magnesium- and phosphate-limited cells exhibited similar levels of resistance to triclosan to planktonic culture. However, carbon-limited cells were massively more resistant if cultured as a biofilm compared with planktonic culture with a surviving fraction of almost 0.1 after 50 min exposure to triclosan. RpoS had some influence on the susceptibility to triclosan in biofilm culture, but only if cells were limited with magnesium or phosphate. No *rpoS*-dependent effects were seen in carbon- or nitrogen-limited cells (fig. 5-18).

Figure 5-10. Influence of carbon limitation on susceptibility to 15 μg / ml triclosan of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} to high- and low-density at 37 °C with aeration (BLD indicates that the surviving fraction was below the level of detection).

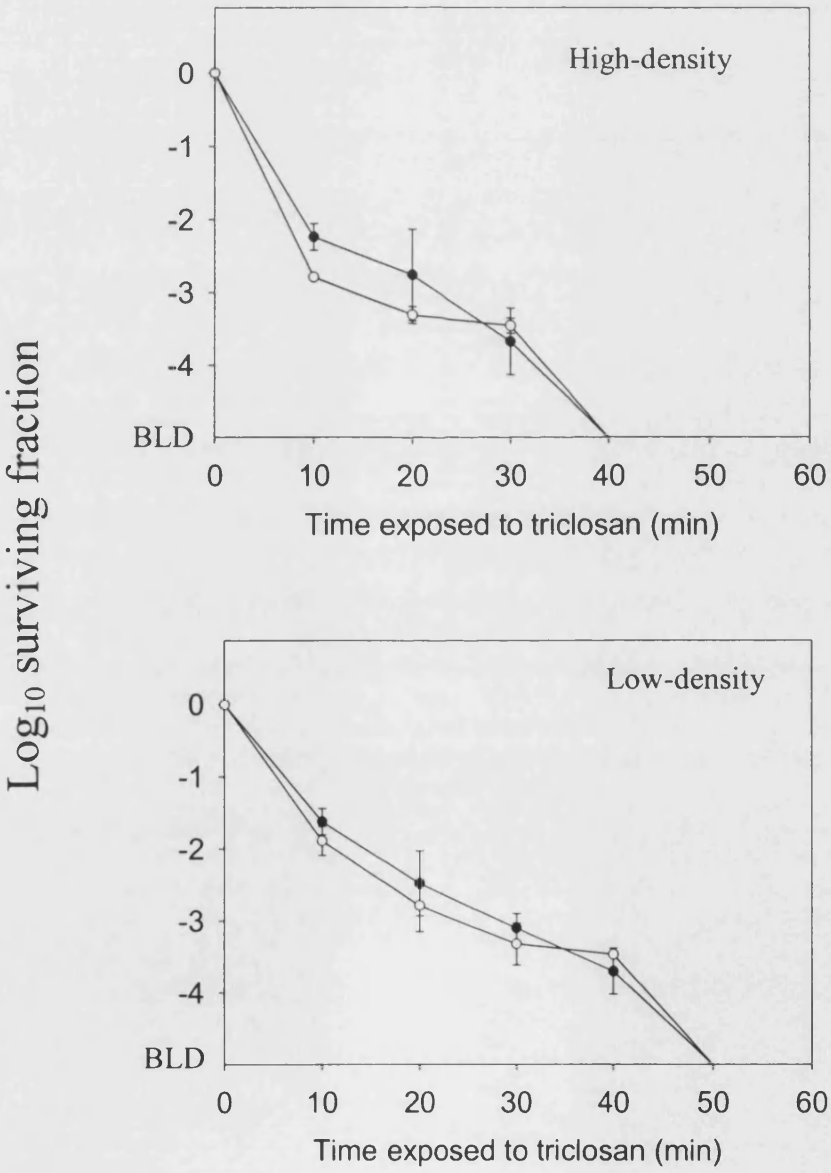


Figure 5-11. Influence of carbon limitation on susceptibility to 15 μg / ml triclosan of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} at 37 °C.

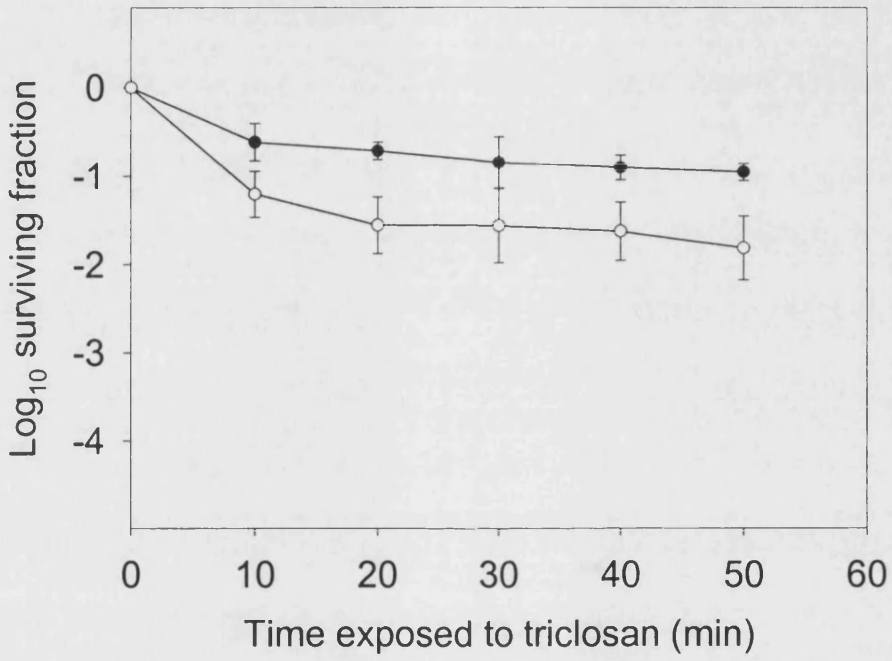


Figure 5-12. Influence of magnesium limitation on susceptibility to 15 μg / ml triclosan of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} to high- and low- density at 37 °C with aeration (BLD indicates that the surviving fraction was below the level of detection).

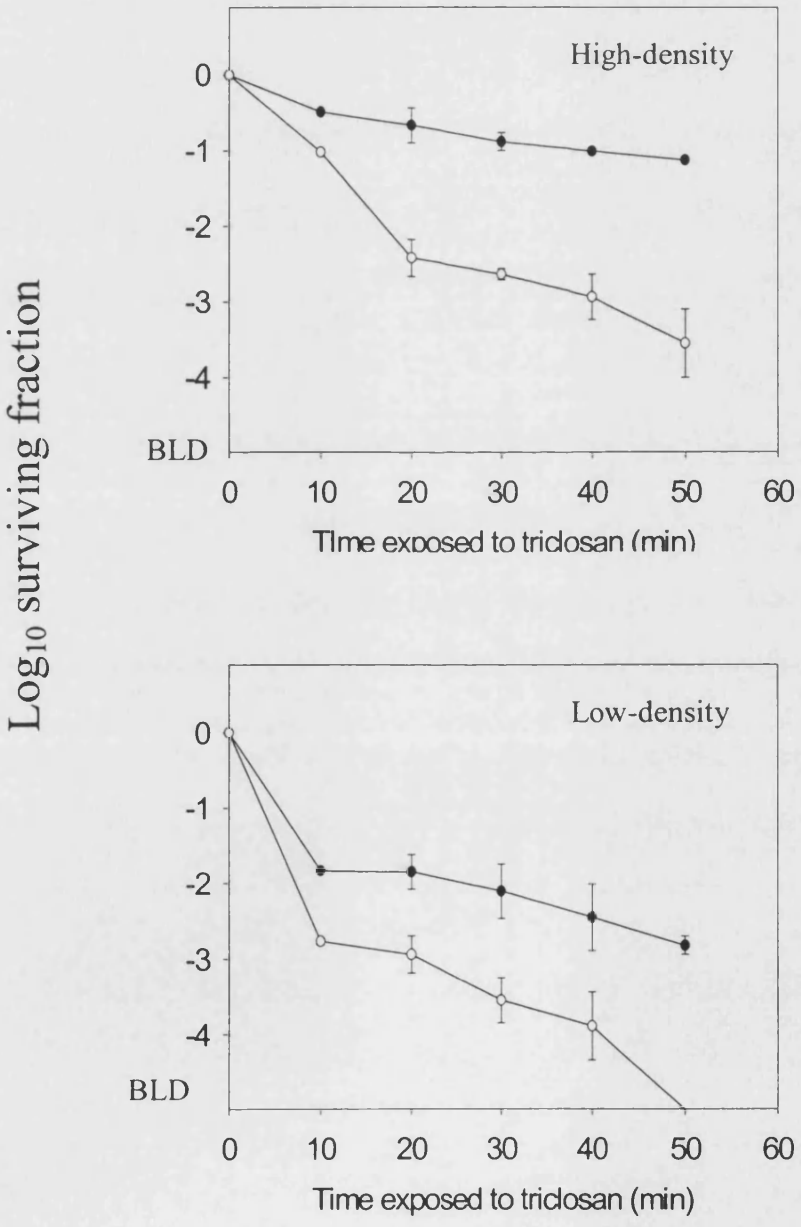


Figure 5-13. Influence of magnesium limitation on susceptibility to 15 μg / ml triclosan of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} at 37 °C.

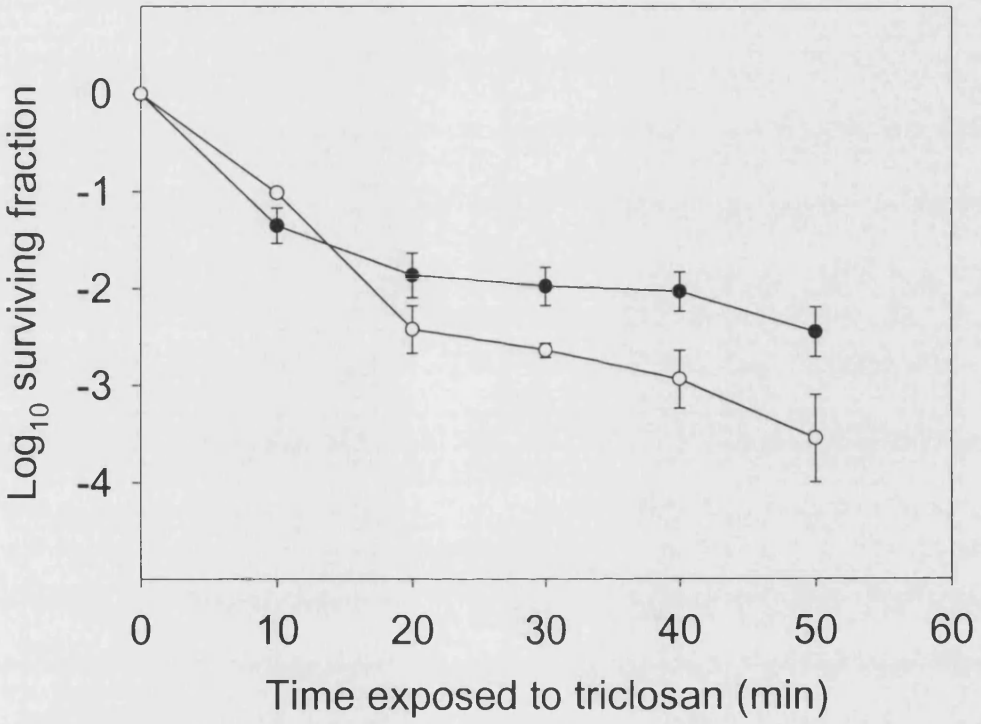


Figure 5-14. Influence of nitrogen limitation on susceptibility to 15 μg / ml triclosan of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} to high- and low-density at 37 °C with aeration.

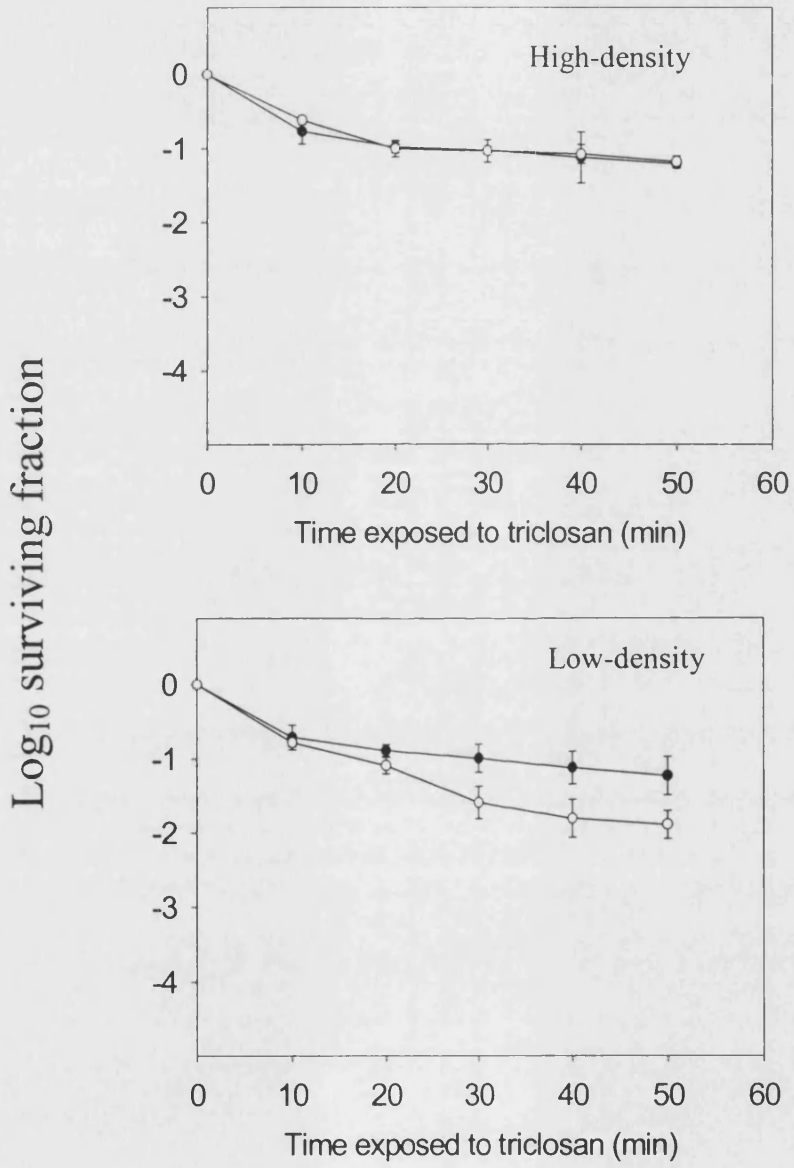


Figure 5-15. Influence of nitrogen limitation on susceptibility to 15 μg / ml triclosan of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} at 37 °C.

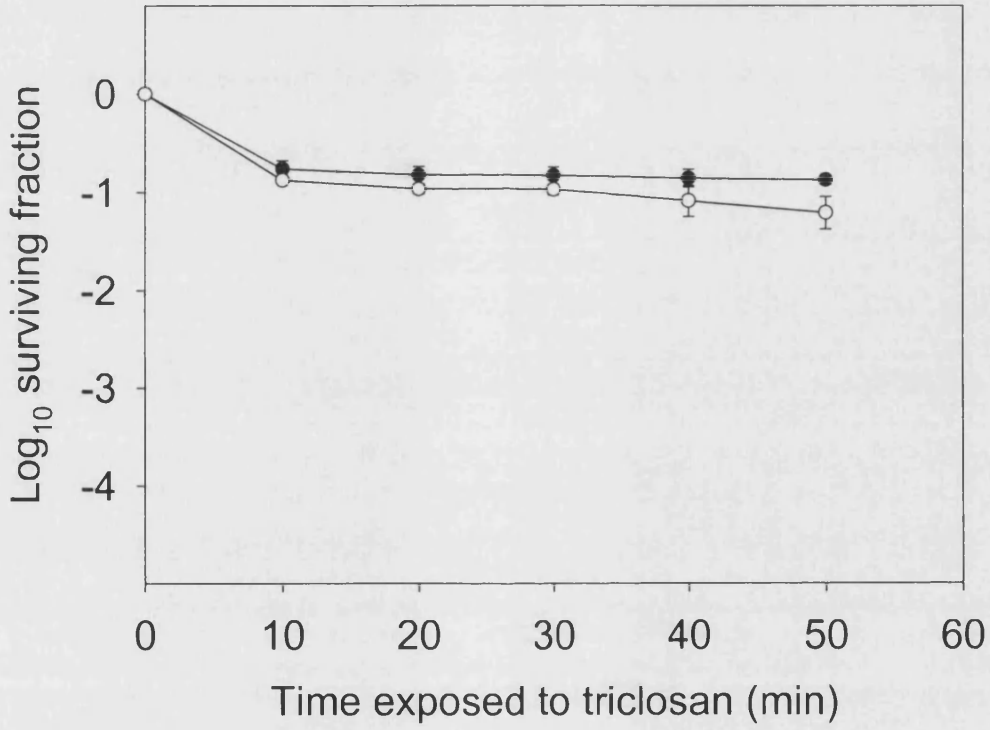


Figure 5-16. Influence of phosphate limitation on susceptibility to 15 μg / ml triclosan of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} to high-and low-density at 37 °C with aeration.

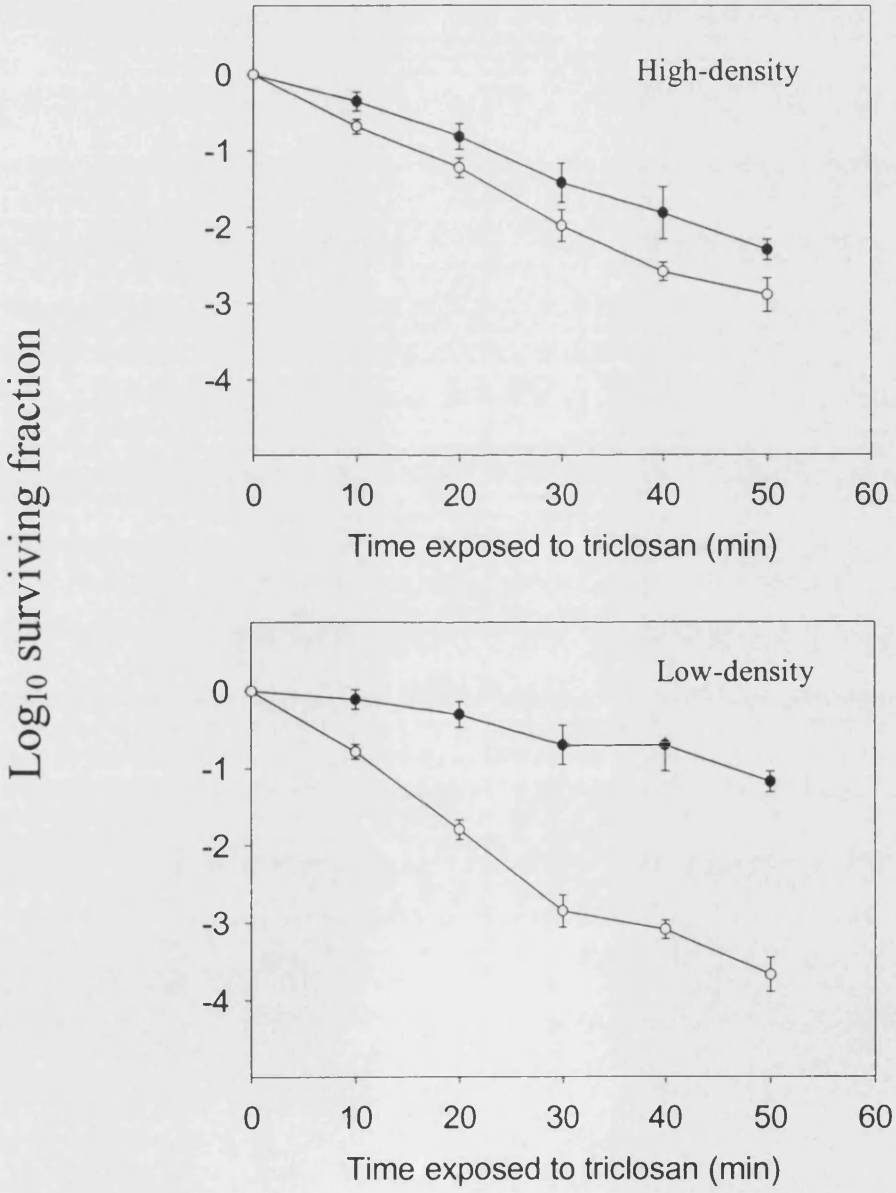


Figure 5-17. Influence of phosphate limitation on susceptibility to 15 μg / ml triclosan of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} at 37 °C.

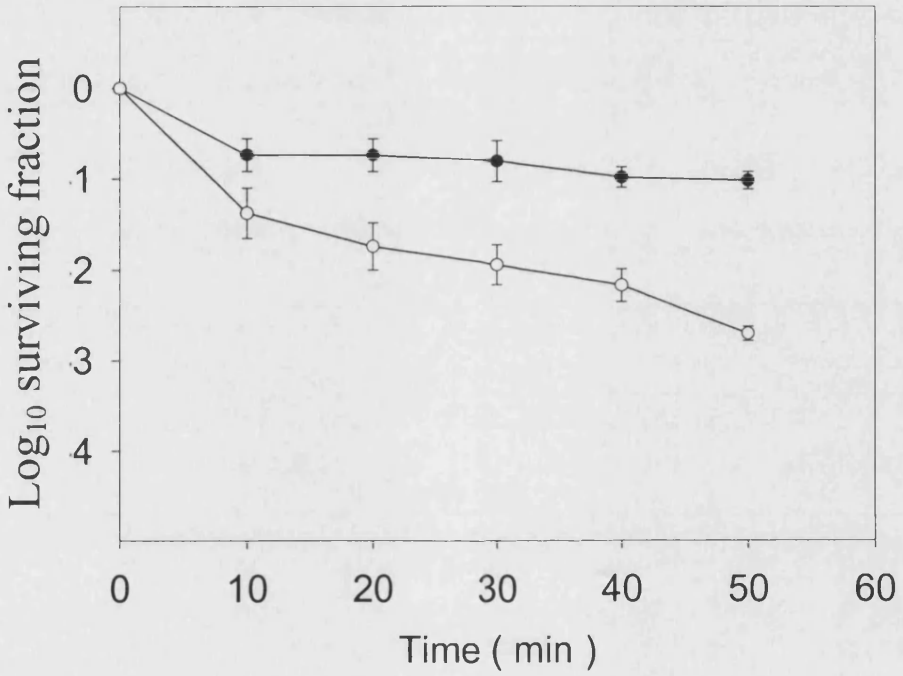
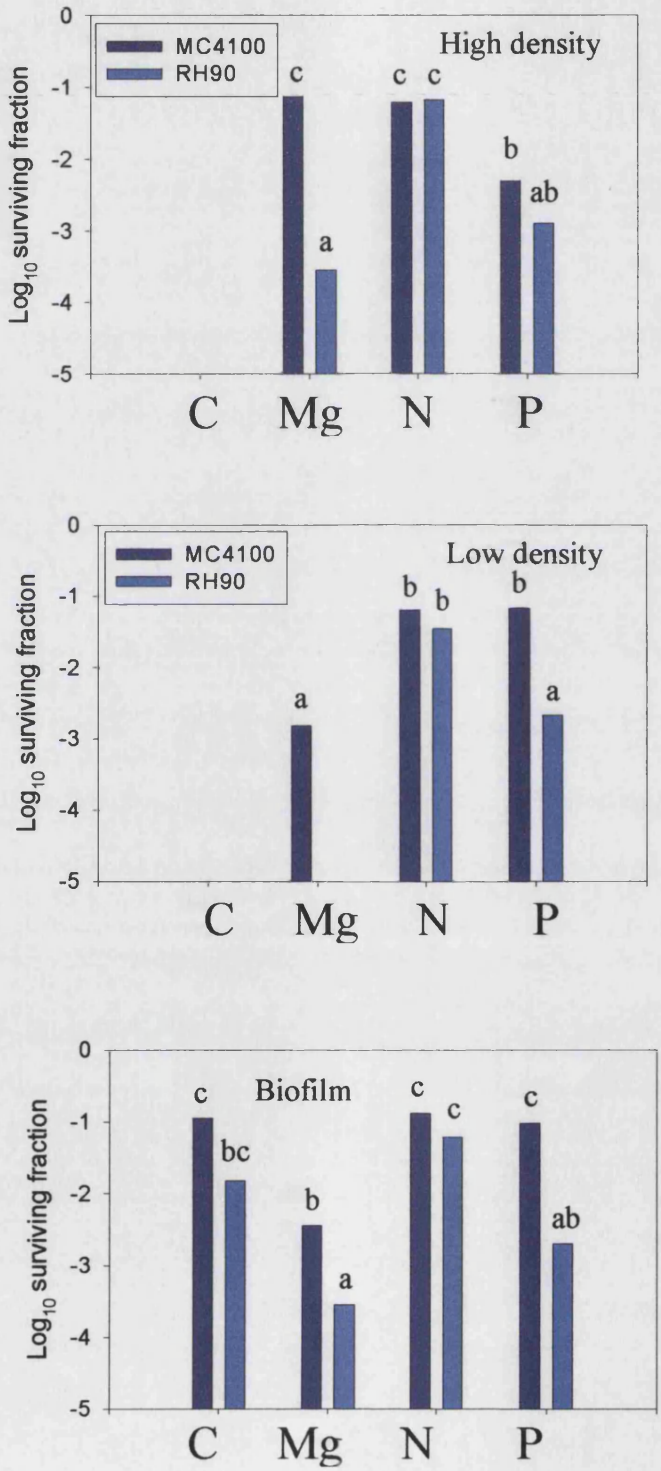


Figure 5-18. Effect of *rpoS* and nutrient limitation on susceptibility to exposure to 15 μg / ml triclosan for 50 min of stationary phase (6 hours after entry) high, low-density planktonic and biofilm *E. coli* MC4100 and RH90.



5.2.3 Effect of *rpoS* and nutrient limitation on susceptibility of *E. coli* to drying at 20 °C

The susceptibility of stationary phase (6 hours after entry) nutrient-limited planktonic and biofilm *E. coli* MC4100 and RH90 (Δ *rpoS*) to desiccation was determined. To ascertain if cell density influenced the susceptibility of *E. coli* to desiccation, planktonic cultures were tested after growth to high- and low-density. The effect of rehydration on viability was examined for planktonic culture. At each time point, a duplicate sample was rehydrated in 1ml of CDM (without the limiting nutrient). Samples were rehydrated for periods of 1 and 24 hours. Rehydration of samples had no influence on cell viability as no more surviving cells were found in the rehydrated samples compared with samples subjected to viable counts immediately (data not shown).

Overall, planktonic cultures were highly sensitive to desiccation as there were no detectable survivors after 5 hours for all nutrient limitations tested (fig. 5-19, 5-21, 5-23, 5-25). However when cells were cultured as a biofilm they were vastly more resistant to desiccation and the surviving fraction decreased by less than one log cycle after 215 hours for all nutrient limitations tested (fig. 5-20, 5-22, 5-24, 5-26).

RpoS had no clear effect on the sensitivity to desiccation. In carbon-limited culture there was no difference in sensitivity between wild type and *rpoS* null mutant cells in biofilm or high-density planktonic cells. However, in low-density planktonic culture, cells lacking *rpoS* (RH90) had detectable survivors one hour longer compared with the wild type strain (MC4100) (fig. 5-19). This pattern was not mirrored by the other nutrient limitations tested. In magnesium- and phosphate-limited culture *rpoS* had no

effect on the sensitivity of biofilm or low-density planktonic cells. However, high-density *rpoS* null mutant cells survived 1 and 2 hours less than the wild type strain for magnesium- and phosphate-limited cells, respectively (fig. 5-21, 5-25). In nitrogen-limited cells *rpoS* had no influence on the sensitivity to desiccation (fig. 5-23).

The bar charts clearly illustrate how highly resistant biofilm cells were compared with the planktonic culture as between 40 and 80 % (depending on the nutrient limitation) survived after 5 hours desiccation if cells were cultured as a biofilm (fig. 5-27). However, there were no detectable survivors after 5 hours desiccation if cells were grown planktonically.

Figure 5-19. Influence of carbon limitation on susceptibility to desiccation of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} to high- and low-density at 37 °C with aeration (BLD indicates that the surviving fraction was below the level of detection).

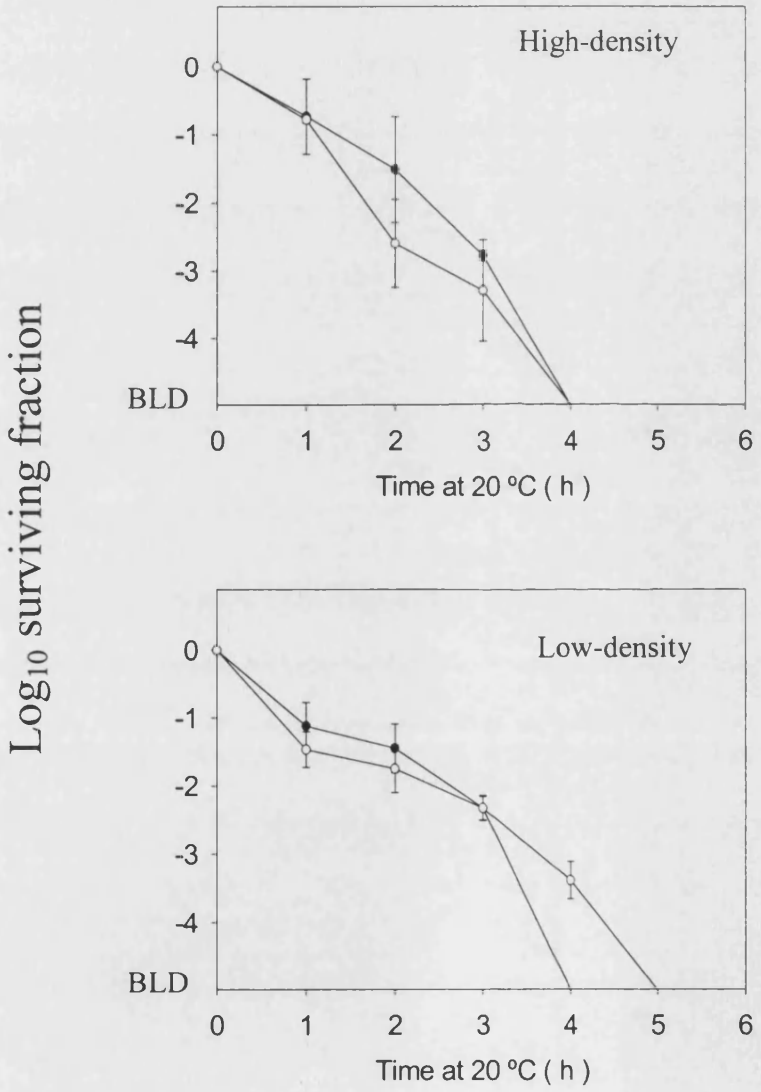


Figure 5-20. Influence of carbon limitation on susceptibility to desiccation of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} with aeration.

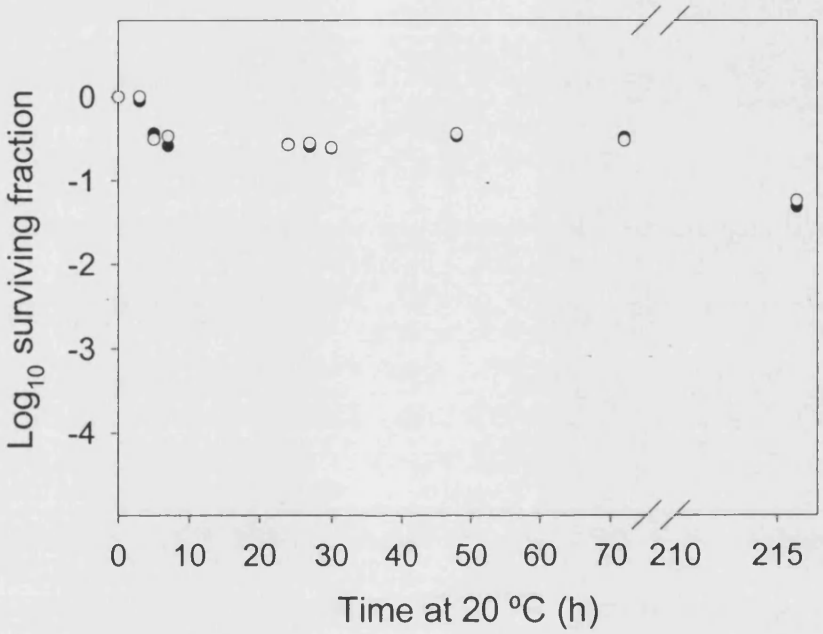


Figure 5-21. Influence of magnesium limitation on susceptibility to desiccation of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} to high- and low-density at 37 °C with aeration (BLD indicates that the surviving fraction was below the level of detection).

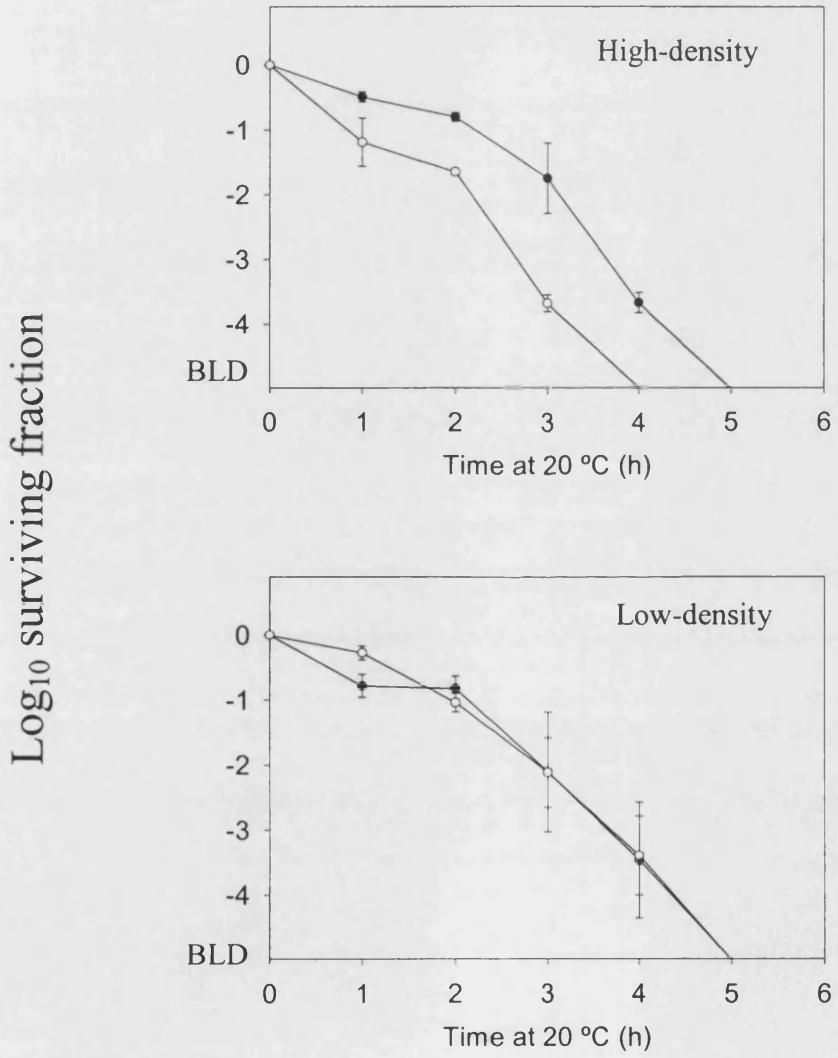


Figure 5-22. Influence of magnesium limitation on susceptibility to desiccation of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} with aeration.

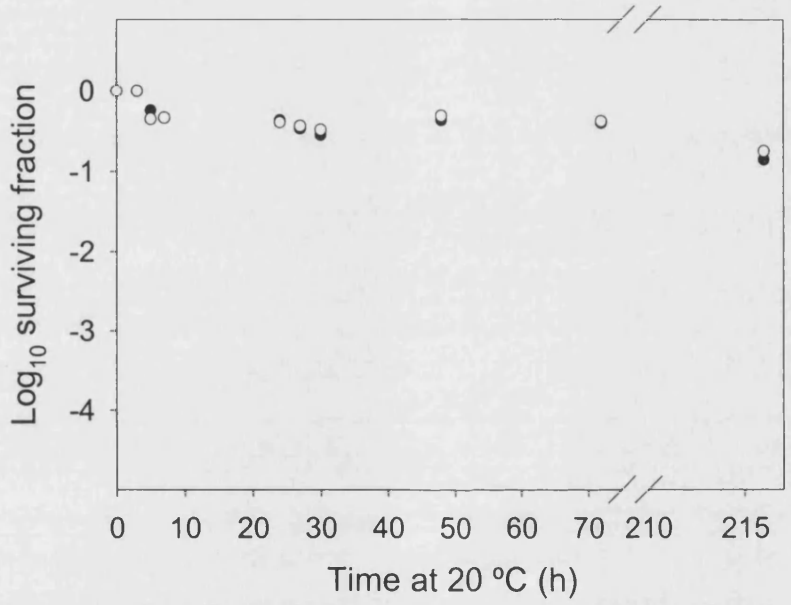


Figure 5-23. Influence of nitrogen limitation on susceptibility to desiccation of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} with nitrogen limitation to high- and low-density at 37 °C with aeration (BLD indicates that the surviving fraction was below the level of detection).

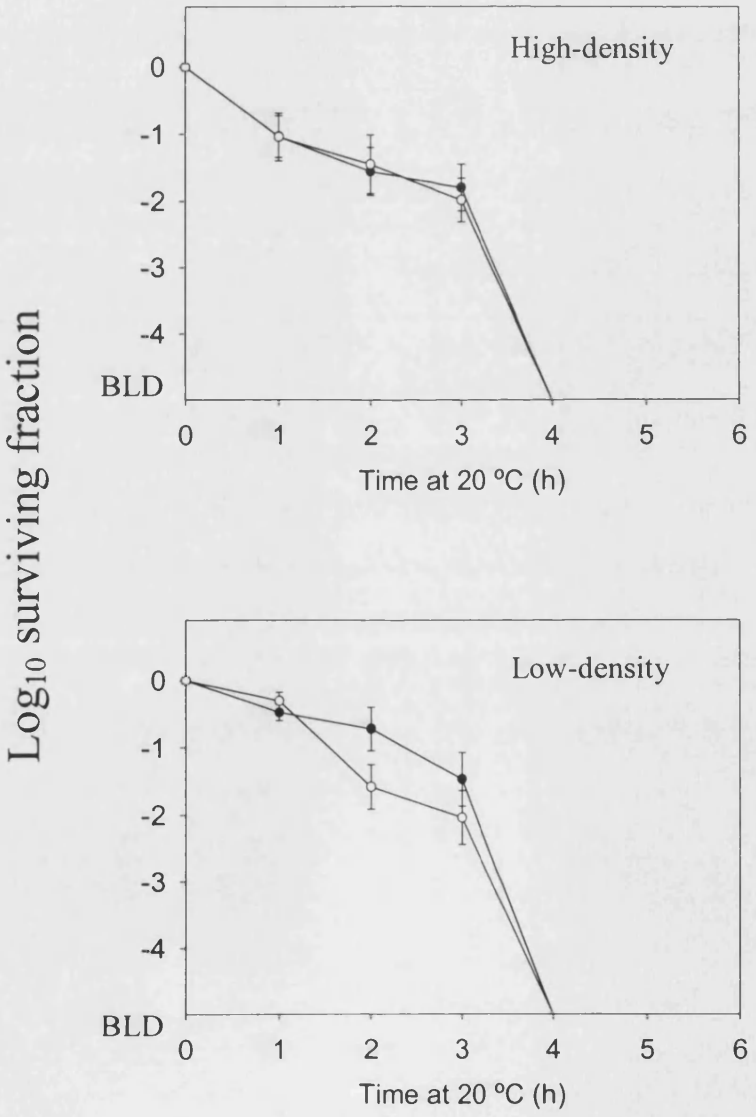


Figure 5-24. Influence of nitrogen limitation on susceptibility to desiccation of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} at 37 °C.

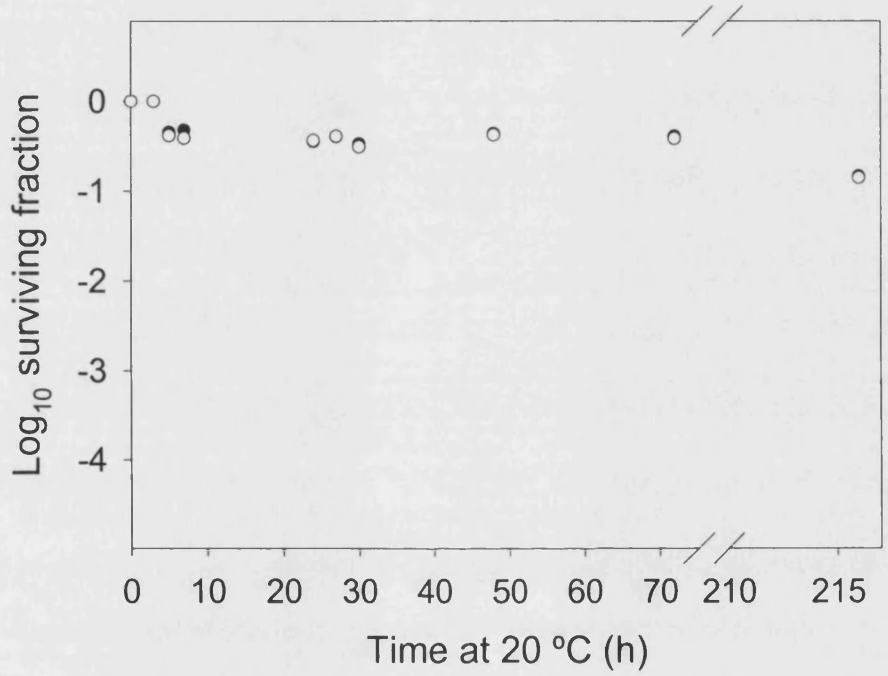


Figure 5-25. Influence of phosphate limitation on susceptibility to desiccation of planktonic stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown in CDM_{10} to high- and low-density at 37 °C with aeration (BLD indicates that the surviving fraction was below the level of detection).

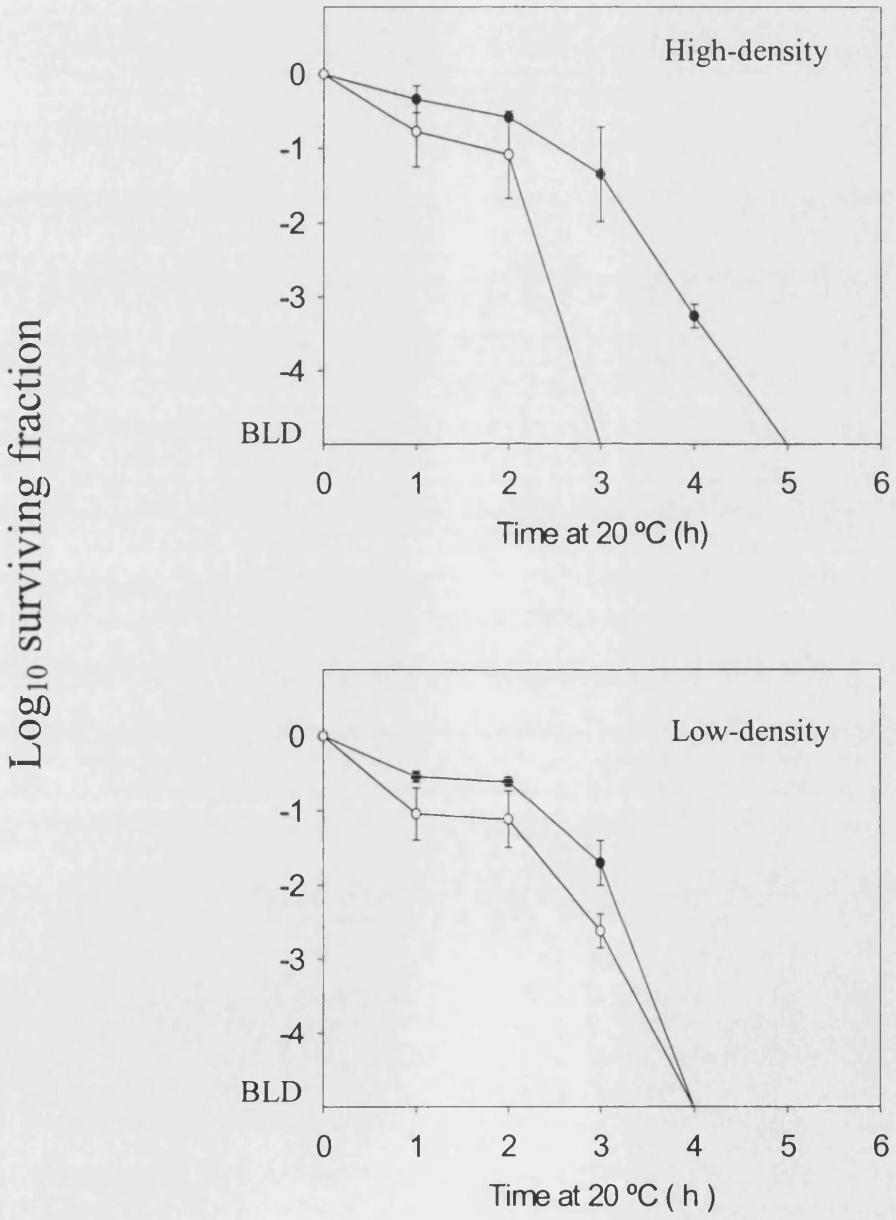


Figure 5-26. Influence of phosphate limitation on susceptibility to desiccation of biofilm stationary phase (6 hours after entry) *E. coli* MC4100 (closed circles) and RH90 (Δ *rpoS*) (open circles) grown on CDM_{10} at 37 °C.

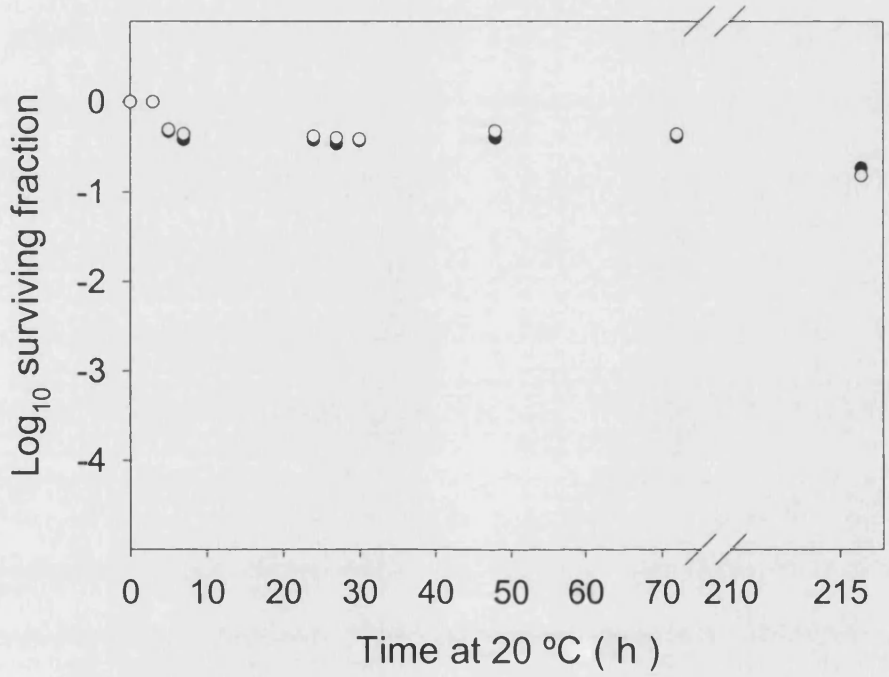
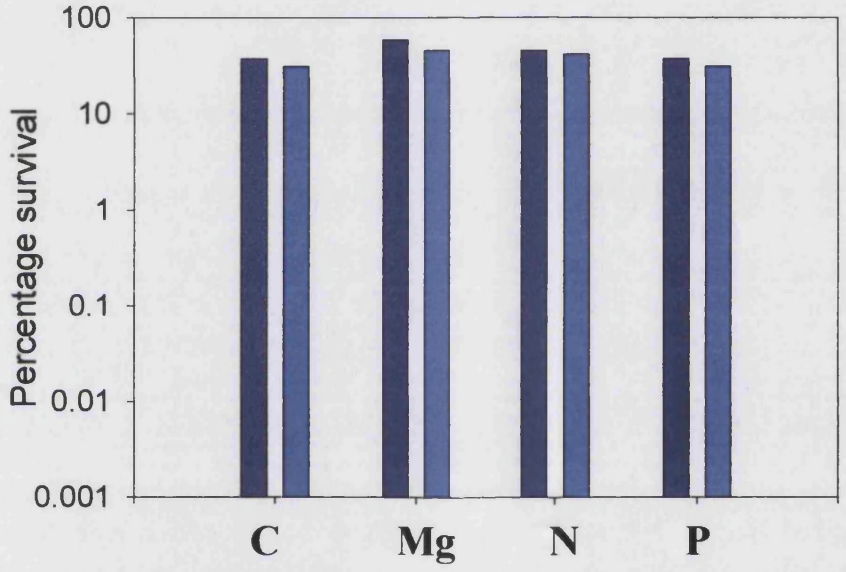


Figure 5-27. Effect of *rpoS* and nutrient limitation on susceptibility to desiccation for 4 h of stationary phase (6 hours after entry) biofilm *E. coli* MC4100 (dark blue bars) and RH90 (Δ *rpoS*) (light blue bars).



5.2.4 Effect of *rpoS* and nutrient limitation on long term stationary phase survival of *E. coli*

The influence of *rpoS* and nutrient limitation on the long-term survival of *E. coli* was examined. Cultures of *E. coli* MC4100 and RH90 were grown planktonically and subjected to nutrient limitation at low- and high-density. The survival of these cultures (at 20 °C) was followed for 74 days by colony count. Viable cells were detected for almost all cultures after 72 days, with the exception of RH90 and MC4100 low-density nitrogen-limited cells.

Examination of the surviving fraction of carbon-limited cultures produced some interesting trends (fig. 5-28). *RpoS* null mutant carbon-limited cultures exhibited a sudden initial drop in viability compared with the wild type. This trend was seen for both low- and high-density carbon-limited cells.

Magnesium-limited cultures showed a different trend of survival compared with carbon-limited cultures. A clear distinction between high and low-density cultures could be seen. Low-density cultures showed approximately 1.5 log cycles greater survival than high-density cultures. Interestingly, *rpoS* appeared to have no effect on the long-term survival of magnesium-limited cultures (fig. 5-29).

When nitrogen-limited cultures were examined, an initial drop in viability of low- and high-density RH90 cells was seen. However, over the remainder of the experiment *rpoS* did not strongly influence the survivability of nitrogen-limited cells. In fact, towards the end of the time course, large density-dependent differences could be seen.

There were no surviving low-density cells after 74 days but survivors were seen for high-density RH90 and MC4100. Interestingly, high-density MC4100 cells showed little decrease in viability over the course of the whole experiment (less than one log cycle) (fig. 5-30).

When the long term survival of phosphate-limited cells is considered it is difficult to ascertain any clear trends in survival. However, wild type (MC4100) showed slightly greater survival between 10 and 50 days compared with the *rpoS* null mutant (RH90), but this trend was not apparent after 50 days (fig. 5-30).

The data from all of the long-term survival studies are all presented in the form of bar charts (fig. 5-31). Overall, it is apparent that the cultures studied survived remarkably well during long-term starvation, as viable cells were found under almost all conditions. It is interesting that the relationship between *rpoS* and long-term survival is complex. Under conditions of carbon and nitrogen limitation, the presence of *rpoS* increased the viability of cells after 74 days starvation (except in low-density nitrogen-limited cells where no survivors were found). However, under conditions of magnesium and phosphate limitation *rpoS* has little effect or was even detrimental to long-term survival.

Figure 5-28. Influence of carbon limitation on the long-term starvation survival of planktonic *E. coli* MC4100 and RH90 (Δ *rpoS*) grown in CDM_{10} to high- and low-density.

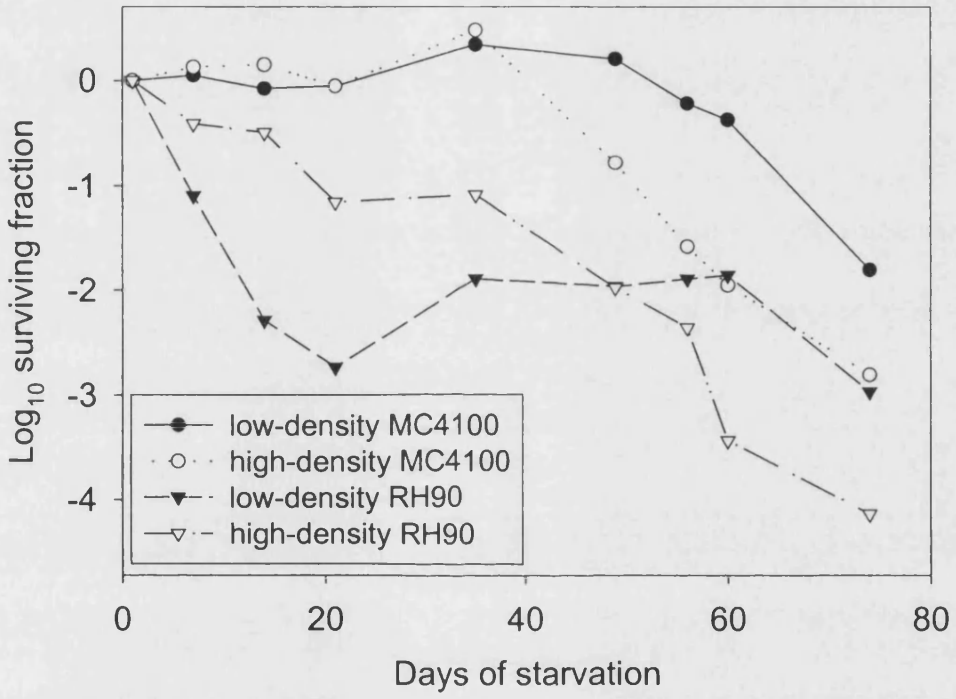


Figure 5-29. Influence of magnesium limitation on the long-term starvation survival of planktonic *E. coli* MC4100 and RH90 (Δ *rpoS*) grown in CDM_{10} to high- and low-density.

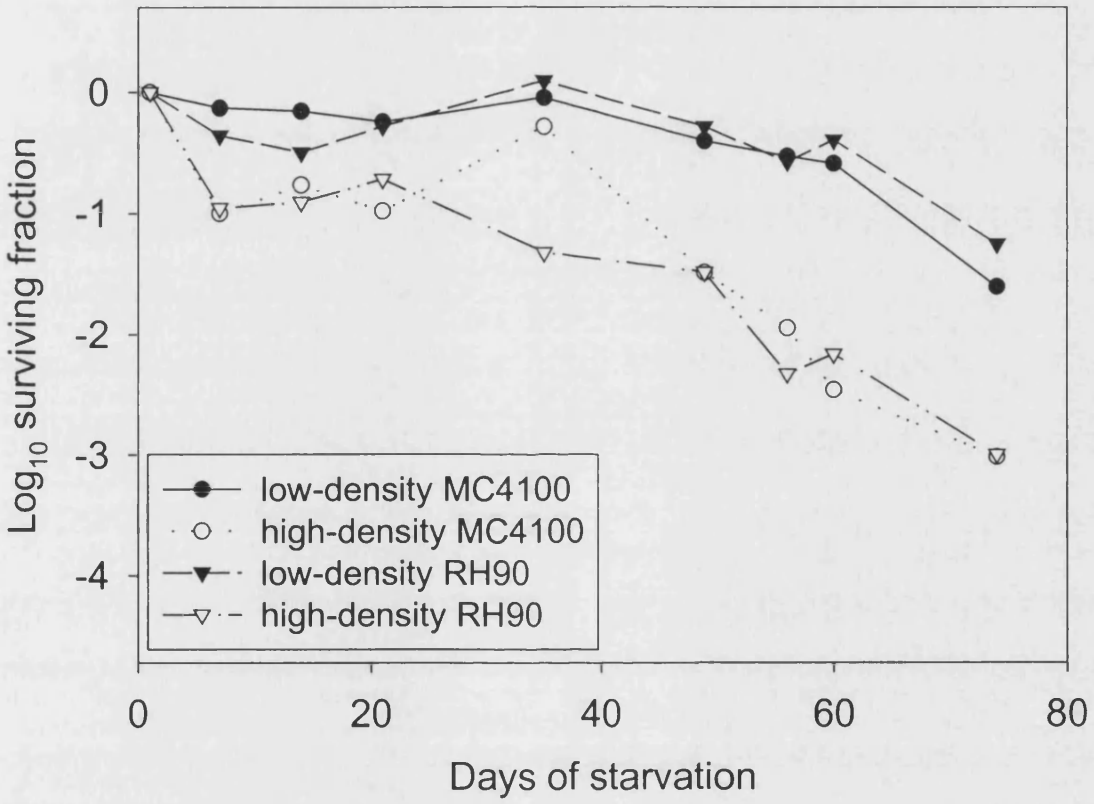


Figure 5-30. Influence of nitrogen limitation on the long-term starvation survival of planktonic *E. coli* MC4100 and RH90 (Δ *rpoS*) grown in CDM_{10} to high and low-density (BLD indicates that the surviving fraction was below the level of detection).

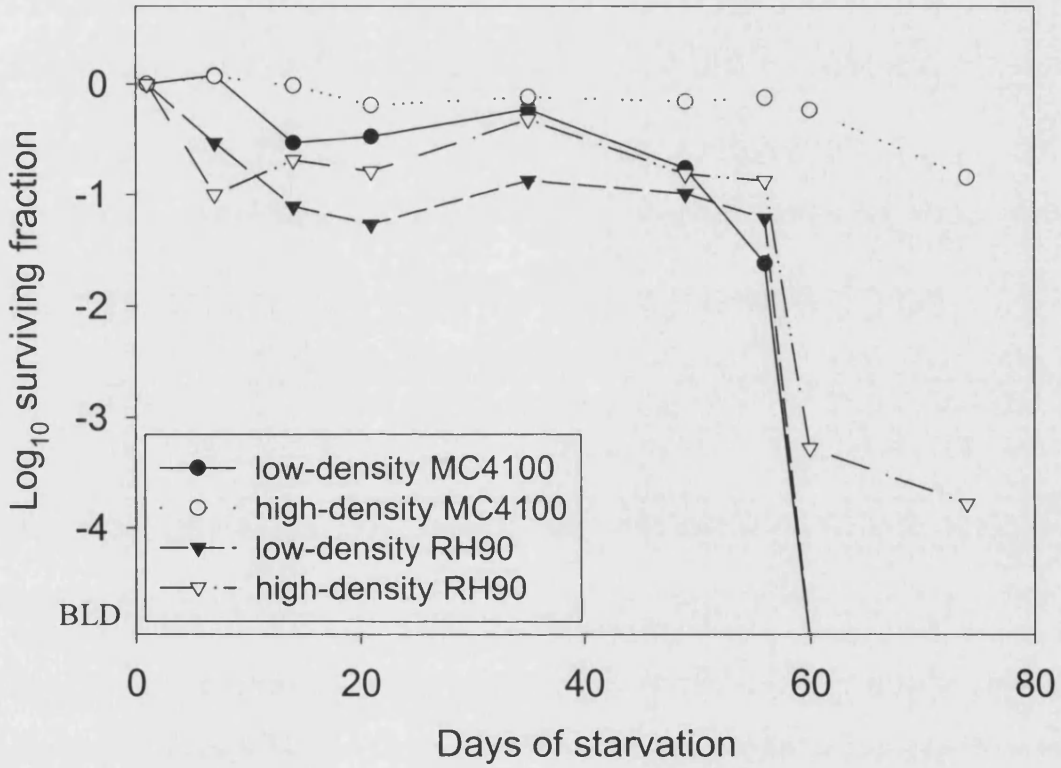


Figure 5-31. Influence of phosphate limitation on the long-term starvation survival of planktonic *E. coli* MC4100 and RH90 (Δ *rpoS*) grown in CDM_{10} to high- and low-density.

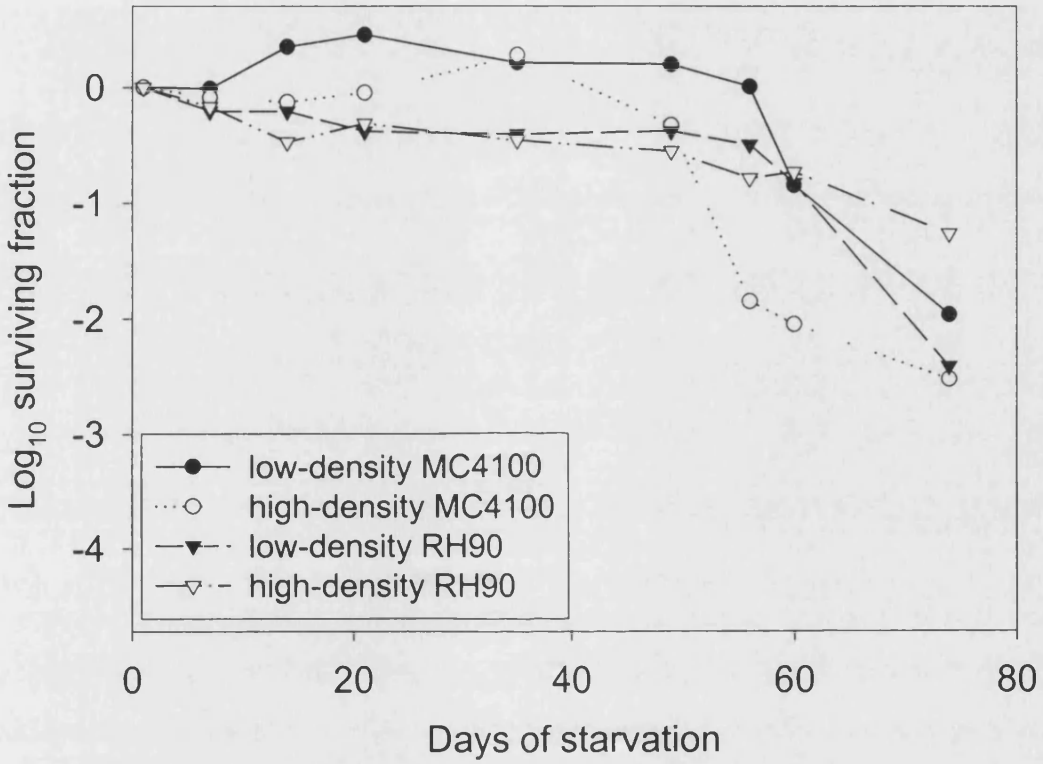
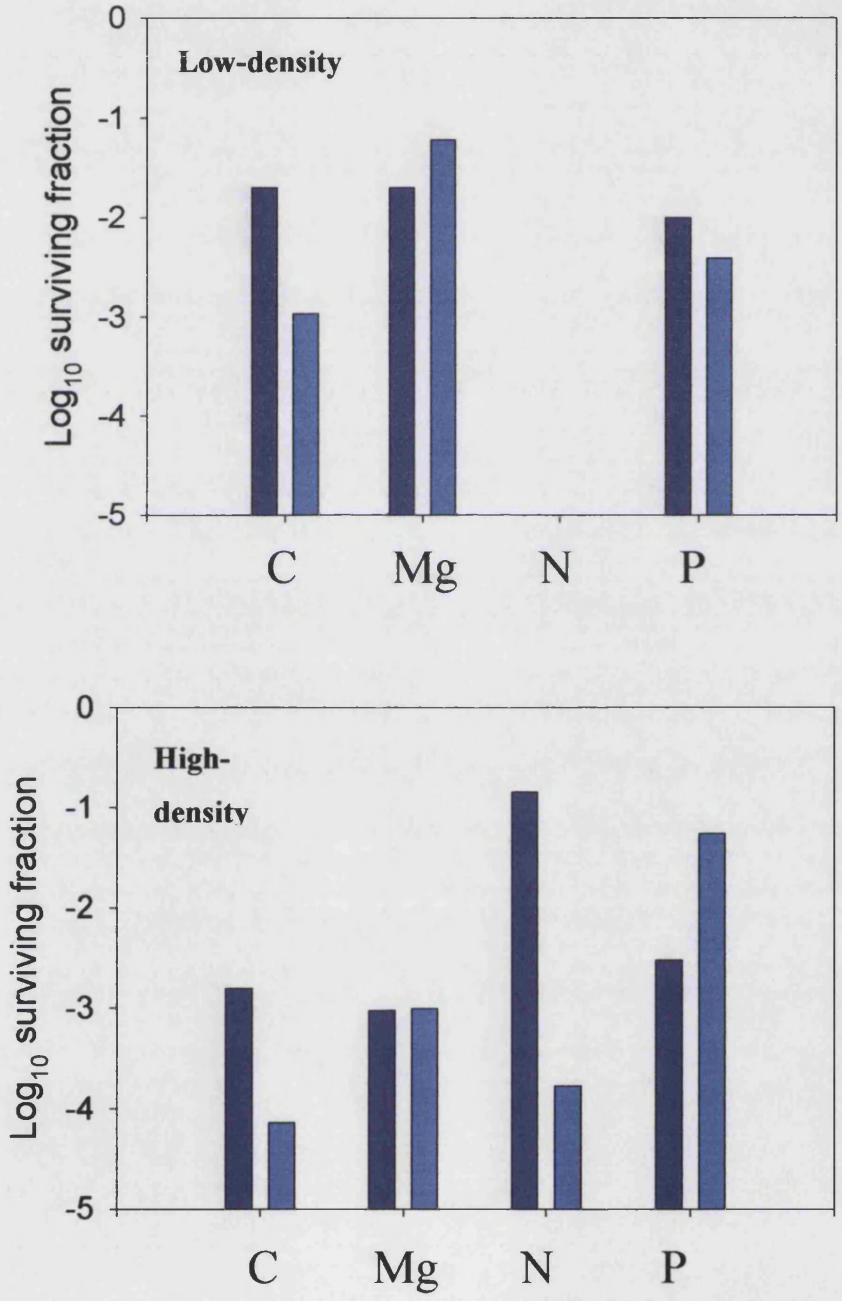


Figure 5-32. Effect of *rpoS* and nutrient limitation on the long-term starvation survival of planktonic *E. coli* MC4100 and RH90 (Δ *rpoS*).



5.3 DISCUSSION

5.3.1 Effect of *rpoS*, nutrient limitation and cell-density on the response of *Escherichia coli* to heat shock

It is difficult to distinguish any clear trends of susceptibility to heat as the pattern varies enormously depending on the growth conditions (the type of nutrient limitation and whether the cells are grown planktonically or as a biofilm) and the strain genotype (whether *rpoS* is present or absent). Previous studies have indicated that the sensitivity of *E. coli* to heat has an element of *rpoS* control as *rpoS* null mutant strains were markedly more sensitive to heat than the corresponding wild type strains (Lange and Hengge-Aronis, 1991). However, as that study was conducted using undefined medium (Lange and Hengge-Aronis, 1991) it is not directly comparable with the findings of this study. If *rpoS* strongly influenced the sensitivity of *E. coli* to heat, carbon- and nitrogen-limited planktonic and all RH90 cultures would have been highly susceptible to heat compared with *rpoS* expressing cells (wild type biofilm cells and magnesium- and phosphate-limited planktonic cells). Planktonic carbon-limited cells were highly sensitive to heat but nitrogen-limited planktonic cells did not exhibit the same pattern and were amongst the most resistant cells tested.

It is not entirely surprising that nitrogen-limited cells were relatively resistant to heat as links between the nitrogen-starvation associated sigma factor σ^{54} (encoded by *rpoN*) and the heat shock response have been proposed previously (Kuczynska-Wisnik et al., 2001; Raina and Georgopoulos, 1991). The *ibpAB* and *pspA* operons are important components of the heat-shock response and are regulated by *rpoN*

(Kuczynska-Wisnik et al., 2001). In addition, the *htrM* gene, which is essential for survival at temperatures in excess of 43 °C contains a *rpoN* consensus sequence at the −12 region of the P2 promoter (Raina and Georgopoulos , 1991) and could therefore be transcribed in response to nitrogen starvation. It is also interesting to note that colanic acid has been implicated in heat tolerance of *E. coli* (Mao et al., 2001) and that this operon is regulated by *rpoN* (Stout and Gottesman, 1990). However, it is thought that colanic acid may be more important in the protection of pathogenic *E. coli* strains to heat and acid stress than non pathogenic organisms (Mao et al., 2001).

If *rpoS* strongly influenced the sensitivity of *E. coli* to heat, the *rpoS* null mutant strain (RH90) would have been more sensitive than the corresponding wild type strain (MC4100). Under certain conditions this phenomenon was indeed seen (high-density magnesium- and phosphate-limited planktonic cells) but in many other cases there was no significant difference between wild type and null mutant cells, or the null mutant cells were more resistant than the wild type. It is not surprising that any *rpoS* regulation of heat tolerance is negated by biofilm growth as other evidence (chapter 4 of this study) has demonstrated that biofilms are physiologically very different from the corresponding planktonic cells. Other authors have long proposed the theory that biofilms cells have phenotypes distinct from planktonic cells (Brown and Barker, 1999; Costerton et al., 1999). The specific response of biofilm cells to heat shock has not been studied previously. It is feasible that colanic acid may also contribute to the resistance of biofilm cells to heat as the *wca* operon (responsible for the production of colanic acid) is strongly upregulated when bacteria are grown as a biofilm (Prigent-Combaret et al., 1999). It is not surprising that the sensitivity of *E. coli* to heat shock is not strongly dependent on *rpoS* as a specific heat shock response exists (Arsene et

al., 2000; Yuru et al., 2000). The σ^{32}/σ^{24} mediated heat shock response would be present and active in strains lacking *rpoS* (RH90); therefore, they could be expected to show some resistance to heat.

Interestingly, some density-dependent effects were seen in the sensitivity of *E. coli* to heat. Overall, high-density cells were more resistant than low-density cells subjected to the same nutrient limitation. This may indicate that quorum sensing plays some role in the resistance of *E. coli* to heat. The idea that quorum sensing may influence the sensitivity of bacteria to stress is not novel. High cell density has been linked to resistance to stress in *Rhizobium leguminosarum* and *Pseudomonas aeruginosa* (Thorne and Williams, 1999; Huang and Shih, 2000). However, this method of protection is conferred by acylated homoserine lactones, which are not present in *E. coli*. In *E. coli* high cell density has been linked to acid resistance although the mechanism is unknown (Datta and Benjamin, 1999). It is feasible that LuxS-mediated quorum sensing could contribute to heat resistance in *E. coli* thus explaining why the high-density cells in this study were overall, more resistant than the corresponding low density cells. In fact, a tentative link between LuxS-mediated quorum sensing and a number of heat shock-associated genes has been made using microarray analysis (DeLisa et al., 2001).

5.3.2 Effect of *rpoS*, nutrient limitation and cell-density on the response of *Escherichia coli* to triclosan

When the effect of exposure to triclosan on *E. coli* was examined some clear trends could be determined. Nutrient limitation had a dramatic effect on the sensitivity of *E. coli* to triclosan (15μg / ml), particularly in planktonic culture. Overall carbon-limited

cells were highly sensitive to triclosan and no viable cells could be recovered after 50 min exposure to triclosan. Nitrogen-limited cells were highly resistant and magnesium- and phosphate-limited cells exhibited intermediate resistance. Many previous studies have demonstrated that nutrient limitation has a profound effect on the sensitivity of bacteria to antimicrobial agents (Cozens and Brown, 1983; Brown, 1977; Gilbert and Brown, 1978; Brown and Melling, 1969).

Most studies have focused on magnesium-depletion and have shown that magnesium-limited cells are massively more resistant than cells that are carbon-limited or grown in complex medium to uncoupling phenols, cetrimide, chlorhexidine and benzalkonium chloride (Cozens and Brown, 1983; Brown and Melling, 1969). It has been proposed that magnesium limitation may cause alterations in the cell envelope that render bacteria more resistant to antimicrobial agents (Cozens and Brown, 1983; Brown, 1977). Interestingly, nitrogen-limited cells proved to be extremely resistant to triclosan in this study. However, the influence of nitrogen limitation on the susceptibility of bacteria to antimicrobial agents has not previously been studied. In addition, the effect of nutrient limitation on the susceptibility of bacteria to triclosan has not previously been studied.

It is possible that colanic acid levels are higher in nitrogen-limited cells (Prigent-Combaret et al., 1999) and that this may act as a physical barrier against the penetration of triclosan into this cell. In a similar manner, there may be copious polysaccharide associated with biofilm cells, which may help prevent triclosan from entering the cell.

It is interesting that *rpoS* appears to affect the sensitivity of *E. coli* to triclosan. Overall, resistance to triclosan was higher under conditions where *rpoS* was expressed, for example, carbon-limited MC4100 cells were much more resistant when grown as a biofilm (where *rpoS* is expressed) and there was a clear difference in sensitivity between wild type and *rpoS* null mutant strains. The only exception to this is in nitrogen-limited cells, which were very resistant, even in the absence of *rpoS* expression. Previously *rpoS* has been shown to confer resistance to a number of antimicrobial agents. Resistance to cephalosporins antibiotics and 1,2-benzisothiazolin was shown to be severely impaired in *rpoS* null mutants (Greenway and England, 1999).

It is feasible that the general protection mechanisms associated with *rpoS* (Hengge-Aronis, 2000) may assist in survival following exposure to triclosan. It is known that triclosan causes non-specific membrane damage and inhibits bacterial fatty acid synthesis at the enoyl-acyl carrier protein reductase (FabI) step. It is possible that *rpoS* regulated events could help prevent membrane damage (in a manner analogous to the prevention of membrane damage in response to ethanol by the *rpoS* regulated *uspB* gene (Farewell et al., 1998)). However, it seems unlikely that *rpoS* could compensate for inhibition of the FabI enzyme, as it requires a missense mutation in this gene to confer resistance to triclosan. It is interesting to note that the pattern of sensitivity to triclosan tends to mirror that of cyclopropane fatty acid content (chapter 4, this study) with more sensitive cells (carbon-limited planktonic culture and RH90) having correspondingly lower levels of cyclopropane fatty acids.

It is known that increased resistance to triclosan can also be mediated by the upregulation of the *acrAB-TolC* multidrug efflux pump (McMurry et al., 1998). It would be interesting to determine if there is any correlation between the level of triclosan sensitivity and the expression of the *acrAB* efflux pump under conditions of nutrient limitation. This will be examined in the subsequent chapter.

In contrast to heat shock, cell density did not have any significant effect on the susceptibility of planktonic *E. coli* to triclosan. The influence of cell density on the susceptibility of *E. coli* to biocides has not previously been studied.

5.3.3 Effect of *rpoS*, nutrient limitation and cell-density on the response of *Escherichia coli* to desiccation

E. coli cultures exposed to desiccation exhibited a remarkable variation in sensitivity depending on the growth conditions. After 5 hours exposure to desiccating conditions no survivors could be detected when cells were cultured planktonically. However, biofilm cells showed only one log cycle decrease in viability after 215 hours of drying. The effect of desiccation on biofilm cells has not previously been studied. It is feasible that the copious exopolysaccharide (EPS) commonly associated with biofilm growth could assist in survival under drying conditions (Pratt and Kolter , 1999; Wai et al., 1998). The effect of EPS on biofilm resistance to drying could be ascertained by using washed resuspended biofilm cells for drying assays rather than testing the biofilm *in situ*. However, it is possible that the action of washing and resuspending biofilm cells itself could constitute a stress that could additionally influence the susceptibility of biofilm cells to drying.

It is possible that the large difference in sensitivity to drying seen in planktonic and biofilm cells could reflect the differences in the test conditions. Planktonic cells were subjected to drying on squares of filter paper whereas biofilms were tested *in situ* on nitrocellulose membranes. It is feasible that the different substrates may lose moisture at varying rates thus affecting the susceptibility of cells to drying. However, it is unlikely that this could account for the 5 log cycle difference in sensitivity seen between planktonic and biofilm cells. This could be tested conclusively by assaying planktonic cells on the same membrane used for biofilms.

This study also examined the influence of *rpoS* on the sensitivity of *E. coli* to drying. RpoS did not affect the sensitivity of biofilms or of low-density planktonic cells to drying. Examination of high-density cells after 3 hours of desiccation indicated that there might be a *rpoS* effect as only wild type magnesium and phosphate-limited cells remained viable. However, this only indicates the differing survivors left after 3 hours and overall there was no difference between *rpoS* wild type and null mutants as no survivors of either strain were present after 5 hours of drying. Interestingly, previous studies have indicated that *rpoS* does play an important role in the sensitivity to drying (Jørgensen et al., 2000). However, this study is not directly comparable to the results presented here as it was conducted using cells grown in rich media and used *Salmonella typhimurium* and not *E. coli*. In addition the strains used were not isogenic wild type and *rpoS* null mutant but natural isolates which showed mutations in *rpoS* (Jørgensen et al., 2000). Therefore, it is feasible that these strains harbored other mutations in addition to *rpoS* that may have influenced sensitivity to drying. Also the test conditions used were much less severe than in this study with 39 % of wild type and 6 % of null mutant cells remaining after 72 hours exposure to

desiccating conditions (Jørgensen et al., 2000). It is feasible that *rpoS* may be beneficial if the desiccation is more prolonged and less acute.

Previous studies have proposed that trehalose is important for survival under desiccating conditions (Leslie et al., 1995). It is apparent from this study that trehalose is not solely responsible for resistance to desiccation as under most nutrient limitations trehalose levels were similar in planktonic and biofilm culture and yet biofilms were dramatically more resistant to the effects of drying. As previous studies have failed to find a correlation between the level of intracellular trehalose and resistance to drying (Welsh and Herbert, 1999) these results are not surprising.

It would be interesting to examine whether the low viability seen with planktonic cells after 5 hours exposure to drying could have been improved by resuspending the cells in some sort of recovery medium before performing viable counts. It is possible that some of the cells had entered a state of extreme dormancy and become viable but non-culturable (VBNC). However, the idea that a VBNC state exists is highly debated and controversial (McDougald et al., 1998; Kell et al., 1998). It is interesting to note that a previous desiccation study found that although no viable *S. typhimurium* were detected using plate counts after 1 weeks exposure to desiccating conditions (Lesne et al., 2001), varying degrees of metabolic activity could be detected using a combination of epifluorescence microscopy and metabolic indicators, suggesting that a sub-population had entered a state where they retained some metabolic activity but were unculturable (Lesne et al., 2001).

5.3.4 Effect of *rpoS*, nutrient limitation and cell-density on the response of *Escherichia coli* to long-term starvation

Studies of the long-term survival of nutrient limited *E. coli* produced some complex trends, initial changes in viability were not reflected in the longer term. No clear patterns of viability could be correlated with *rpoS*, nutrient limitation or cell density. Although this experiment was only followed in its entirety once, a replicate study that analyzed the survival of cells after 74 days produced very similar results.

One finding that is apparent from this study is that *rpoS* is not essential for long-term survival under conditions of starvation. These results are corroborated by a previous study that found that although *rpoS* was beneficial in short-term survival during starvation, *rpoS* null mutants actually survived better during long-term starvation (Conter and Gangneux et al., 2001). It is not entirely surprising that *rpoS* is not required for long term survival as it is known that natural isolates of *E. coli* frequently exhibit mutations in *rpoS* (Jishage and Ishihama, 1997). The natural environment is likely to show some similarity to the conditions employed in this study as nutrients are generally scarce and rapid exponential growth unusual (Kolter, 1999).

Although the precise physiological consequences of *rpoS* mutation have not been studied in the natural environment, long-term laboratory studies have demonstrated that mutations in *rpoS* produce a distinct growth advantage during stationary phase (GASP) phenotype (Zambrano et al., 1993). Partial loss-of-function mutations in *rpoS* confer the ability to out-compete the parental wild type during long-term starvation (Zambrano et al., 1993). Interestingly, studies of the GASP phenotype have indicated that some limited *rpoS* function is required as a null allele of *rpoS* does

not confer a GASP phenotype over a wild type parent (Finkel et al., 2000). It would be interesting to analyze further the wild type cultures used in this study following long-term starvation and ascertain whether *rpoS* is still functional or if there has been selection for (partial) loss-of-function. In addition, it is important to note that studies of the GASP phenotype have been conducted using undefined media (Finkel et al., 2000) and it would clarify matters to perform competition experiments in CDM with nutrient limitation.

6 EFFECT OF EXPOSURE TO ANTIMICROBIAL AGENTS, DOMESTIC PRODUCTS AND FOOD STUFF ON THE EXPRESSION OF *RPOS*, *ACRAB* AND *MARRAB*

6.1 INTRODUCTION

Recently, there has been widespread concern that exposure of bacteria to biocides in the domestic environment may lead to multiple antibiotic resistance (reviewed in Russell, 1999; Schweizer, 2001). Concern that biocide usage may lead to antibiotic resistance began with the finding that exposure of *E. coli* to pine oil led to the selection of mutants resistant to multiple antibiotics including tetracycline, chloramphenicol, nalidixic acid (Moken et al., 1997). Subsequently, it was shown that this resistance was mediated by the multiple antibiotic resistance (*marRAB*) operon and the *acrAB* efflux pump (Moken et al., 1997). Recently, much research has focused on the widely used biocide triclosan and its effect on *marRAB* and *acrAB* (McMurry et al., 1998).

The *marRAB* operon controls multiple antibiotic resistance in *E. coli* by the production of MarA (Gambino et al., 1993; Cohen et al., 1988), a protein with high homology to members of the XylS / AraC family of transcriptional activator proteins (Alekshun and Levy, 1997). MarA alters the expression of several unlinked target genes including the stress-induced efflux system *acrAB* (Ma et al., 1995) and *micF,* an antisense RNA involved in the down regulation of the outer membrane porin F (OmpF) (Cohen et al., 1989; Cohen et al., 1988). The repressor MarR, encoded by *marR*, binds to the *marO* operator region to regulate negatively expression of *marRAB* (Seone and Levy, 1995; Sulavik et al., 1995). The function of MarB is unknown. A

number of structurally unrelated compounds including chloramphenicol, tetracycline, menadione, paraquat, plumbagin and sodium salicylate have been shown to induce *marRAB* (Cohen et al., 1993; Seone and Levy, 1995). In addition to its role in antibiotic resistance, *marRAB* is also involved in resistance to weak acids and organic solvents (White et al., 1997; Ariza et al., 1994).

The *acrAB* efflux pump has been identified as the major mechanism responsible for multi-drug resistance in multiple antibiotic resistant mutants (Okusu et al., 1996; Sulavik et al., 2001). The AcrAB efflux pump has wide substrate specificity and is involved in the efflux of numerous structurally unrelated compounds (Ma et al., 1995). The AcrAB system comprises of AcrA a membrane fusion protein (MFP) that acts as a periplasmic accessory protein and AcrB an inner membrane transporter of the RND (resistance nodulation cell division) family (Zgurskaya and Nikaido, 1999). However, the *acrAB* operon lacks the gene for the outermembrane component of the efflux pump and recruits TolC, a multifunctional outermembrane channel to complete the tri-partite complex (Fralick, 1996)

The aim of this study was to determine whether triclosan (and other widely used antimicrobial agents) could cause induction of *acrAB* and *marRAB* and also if they could induce expression of *rpoS*. It was also decided to ascertain whether exposure of *E. coli* to other compounds commonly found in the domestic environment such as domestic cleaning products and food could induce *acrAB*, *marRAB* and *rpoS*.

6.2 RESULTS

6.2.1 Expression of *rpoS* in response to antimicrobial agents

The expression of *rpoS* following exposure to the antibiotics tetracycline and norfloxacin and the biocides triclosan and benzalkonium chloride was examined. Expression of *rpoS* was monitored using a late-translational β- galactosidase reporter fusion (RO91) (Lange and Hengge-Aronis, 1994). β- galactosidase activity was measured according to the method of (Miller J, 1972) and expressed in Miller units. *RpoS* expression was examined in cells challenged with antimicrobial agents in exponential (4 generations before the onset of stationary phase) and stationary phase (2 hours after entry). As no expression was found in response to exposure to antimicrobial agents during stationary phase, the results are not presented here. Expression of *rpoS* was examined in high-density planktonic carbon-limited cells. Carbon-limited cells were chosen as expression could not be monitored in magnesium or phosphate-limited cells as the naturally high induction of *rpoS* at the onset of stationary phase would mask any *rpoS* induction caused by exposure to antimicrobial agents.

RpoS expression was induced in response to tetracycline, but only at the second highest concentration tested (0.025 μg / ml). In response to this concentration of tetracycline *rpoS* induction was three times the basal level. Interestingly, this was the lowest concentration of tetracycline tested that perturbed the growth rate of *E. coli* RO91 (figure 6-1).

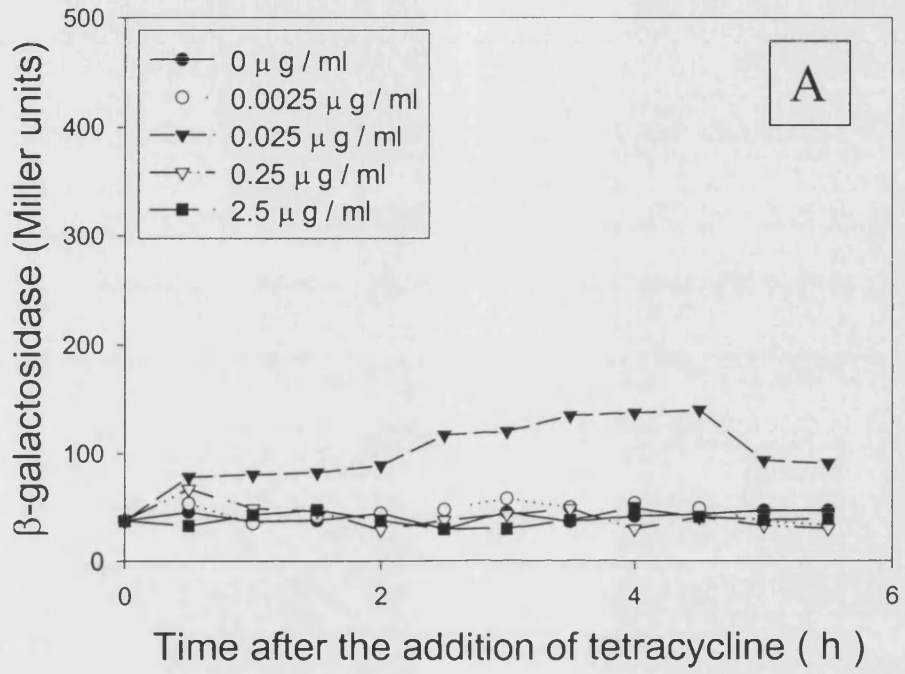
In response to exposure to norfloxacin *rpoS* was induced at least two-fold at all concentrations tested. The highest level of induction was seen with the lowest

concentration of norfloxacin tested (0.005 μg / ml), where expression increased five-fold. At higher concentrations of norfloxacin growth was dramatically curtailed and *rpoS* expression was not induced strongly (figure 6-2).

Exposure to the biocide benzalkonium chloride (BKC) produced a different pattern of *rpoS* expression. Expression of *rpoS* was induced at all concentrations of BKC tested and was proportional to the concentration used. Expression was highest with 1×10^{-4} % and was in excess of six times the basal level. Interestingly, exposure to BKC did not cause perturbations in growth rate analogous to those seen with norfloxacin and tetracycline. Rather than causing the growth rate to decrease dramatically, exposure to BKC only decreased growth rate marginally and caused cells to cease growing at a lower density in a manner comparable with graded nutrient limitation (figure 6-3).

Exposure to triclosan produced particularly dramatic results in terms of *rpoS* expression. In response to 0.1 μg / ml of triclosan *rpoS* expression increased over twenty-fold. Induction of *rpoS* was also seen with the lower concentrations of triclosan tested, with the exception of 0.01 μg / ml. This concentration had no effect on the growth of *E. coli* RO91 (figure 6-4).

Figure 6-1. Effect of exposure to tetracycline during exponential phase on (A) the expression of *rpoS* and (B) the growth of *E. coli* RO91 (*rpoS*::lacZ) in carbon-limited CDM at 37 ° C with aeration (curves are off set for clarity).



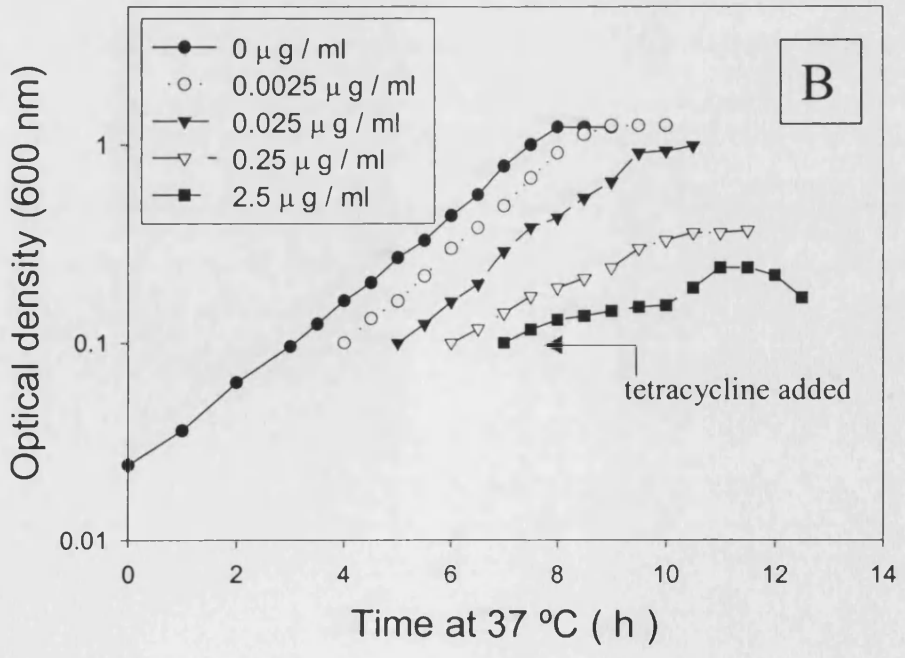


Figure 6-2. Effect of exposure to norfloxacin during exponential phase on (A) the expression of *rpoS* and (B) the growth of *E. coli* RO91 (*rpoS*::lacZ) in carbon-limited CDM at 37 ° C with aeration (curves are off set for clarity).

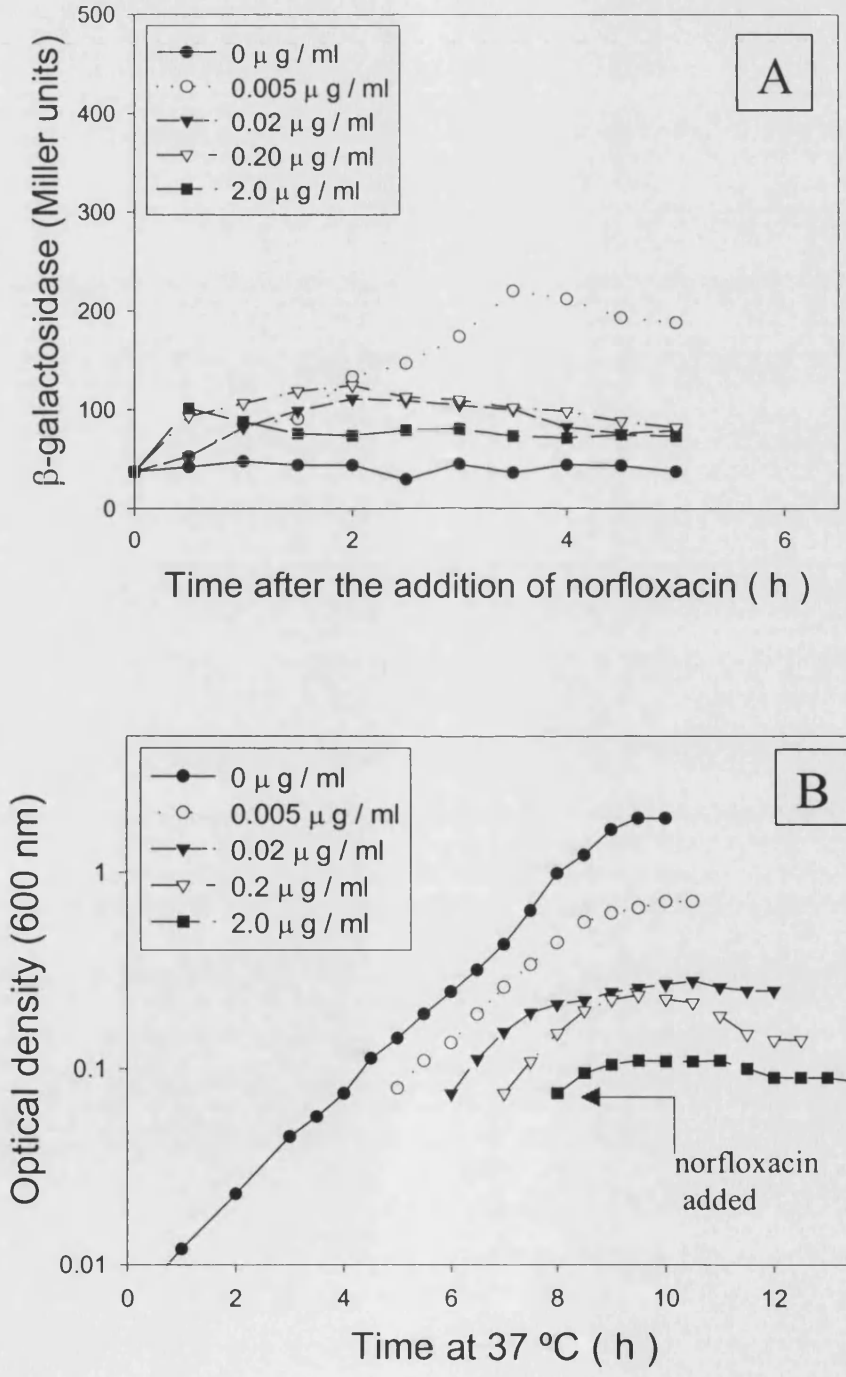
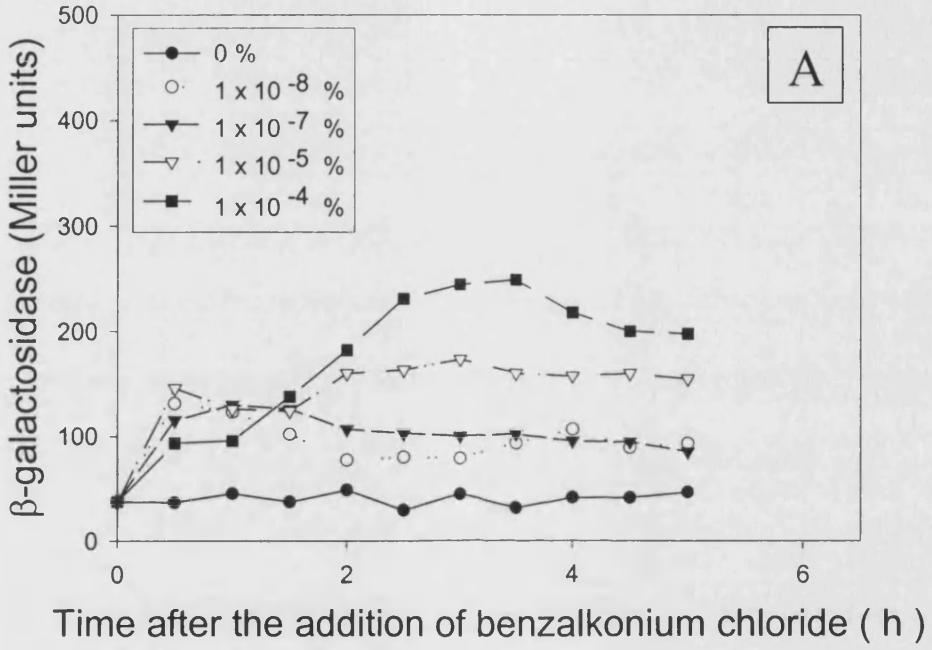


Figure 6-3. Effect of exposure to benzalkonium chloride during exponential phase on (A) the expression of *rpoS* and (B) the growth of *E. coli* RO91 (*rpoS*::lacZ) in carbon-limited CDM at 37 ° C with aeration (curves are off set for clarity).



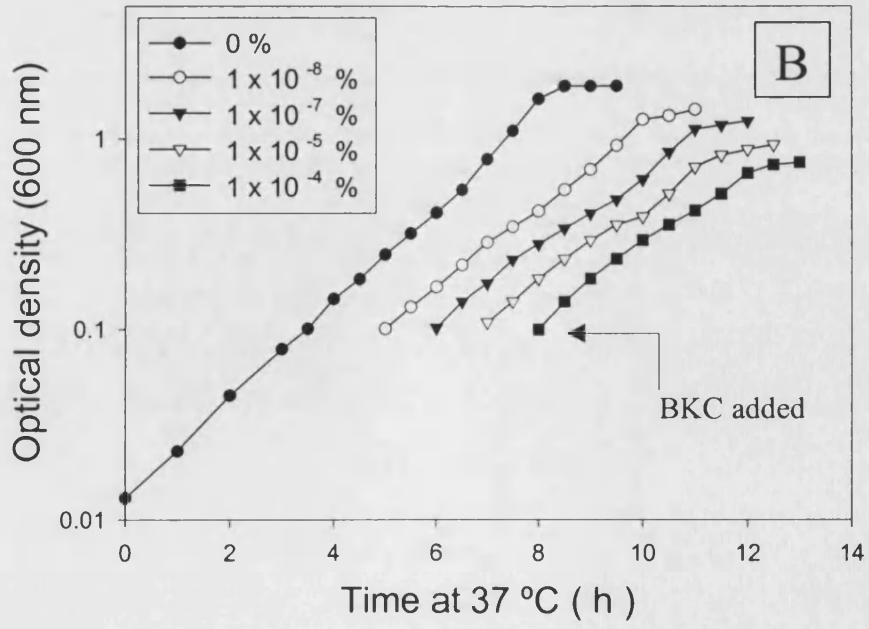
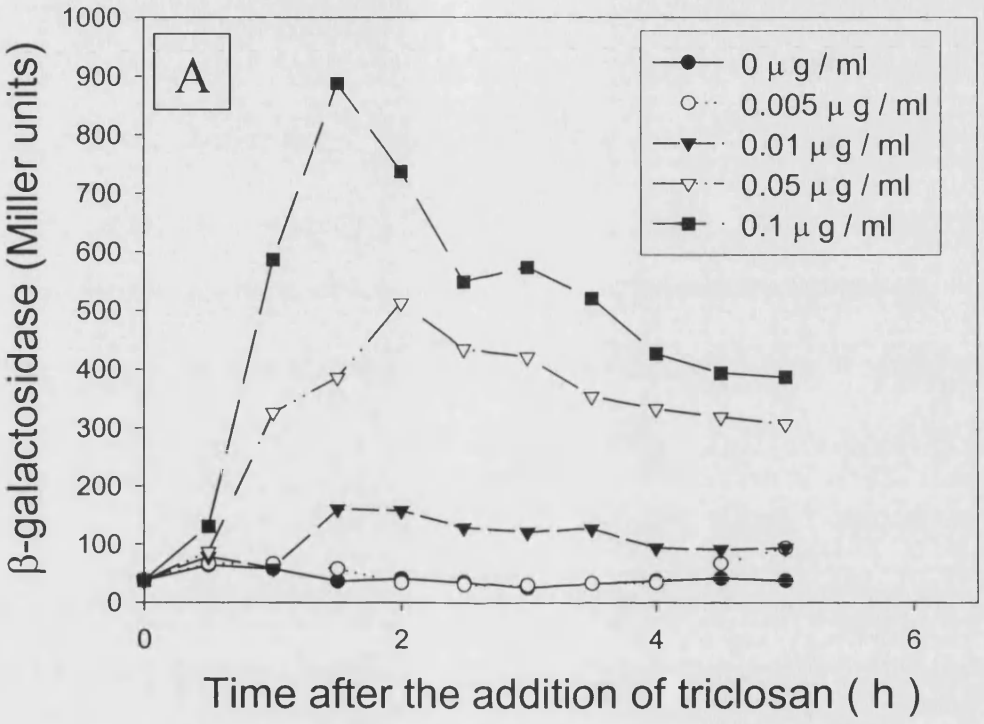
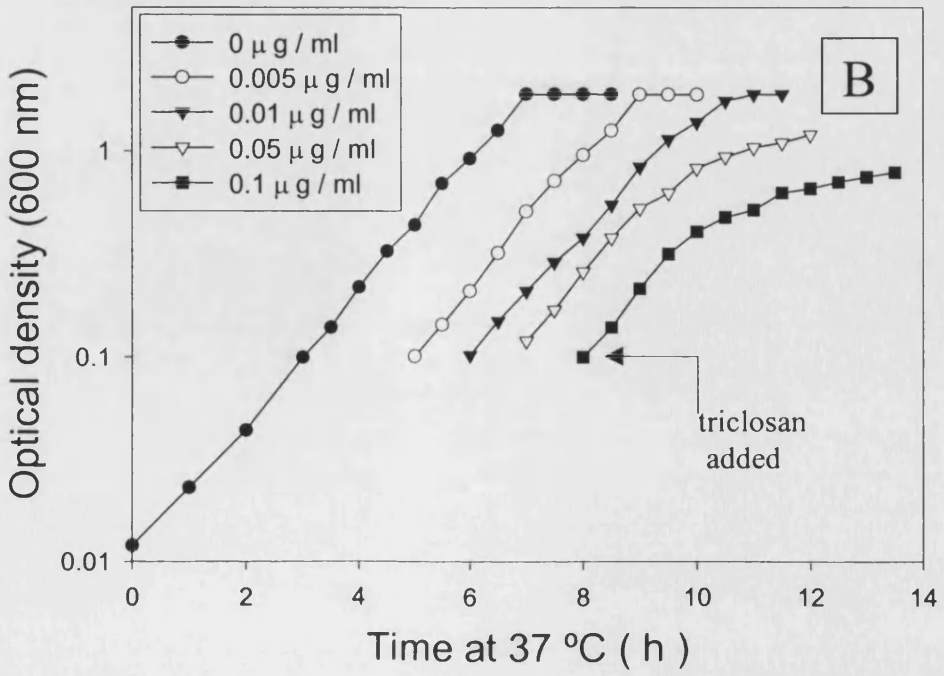


Figure 6-4. Effect of exposure to triclosan during exponential phase on (A) the expression of *rpoS* and (B) the growth of *E. coli* RO91 (*rpoS*::lacZ) in carbon-limited CDM at 37 ° C with aeration (curves are off set for clarity).





6.2.2 Expression of *marRAB* in response to nutrient limitation and exposure to chemical agents

The expression of the *marRAB* operon in response to nutrient limitation was examined using a β-galactosidase reporter fusion to *marRAB* (B306) (Gambino et al., 1993). The expression of *marRAB* was examined in response to carbon, magnesium, nitrogen and phosphate limitation in CDM at both high- and low-density. Only the results obtained with high-density nutrient limited CDM are presented here. However, very similar results were obtained when cells were subjected to nutrient limitation at low-density. The expression of *marRAB* was very low (less than 10 Miller units) in response to all of the nutrient limitations tested and showed no increase upon entry into stationary phase (figures 6-5, 6-6). The expression of *marRAB* was then examined in complex, Luria-Bertani medium and again found to be low with no increase throughout the growth cycle (figure 6-7). *MarRAB* expression was then tested in response to salicylate. Sodium salicylate is referred to as the 'gold standard' for *marRAB* induction as it is the strongest inducer of *marRAB* expression known (Cohen S P et al., 1993). However, when cells were grown in carbon-limited CDM there was no induction of *marRAB* in response to exposure to salicylate. Exposure to all of the salicylate concentrations tested resulted in a dramatic perturbation of growth rate (figure 6-8). When cells were grown in LB broth, salicylate strongly induced *marRAB* expression (figure 6-9). *MarRAB* induction increased 6 –fold even if there was no alteration in growth rate (0.5 mM salicylate). Higher concentrations of salicylate (5.0 mM) resulted in massive *marRAB* expression, to a peak of over 22 times the basal level. However, growth rate decreased concomitantly at this concentration.

The expression of *marRAB* in response to triclosan and ethanol was then studied. Triclosan was chosen, as it is known to cause the induction of *rpoS* (this study) and ethanol as it induces the *marRAB*-dependent, *acrAB* efflux pump (Ma et al., 1995). The expression of *marRAB* was studied after exposure to triclosan (0.1 μg / ml) and ethanol (4 %) in exponential cells grown in carbon-limited CDM and LB broth. No induction of *marRAB* occurred in either CDM or LB grown cells (figure 6-10, 6-11).

Figure 6-5. Expression of *marRAB* (open circles) throughout the growth cycle (closed circles) for planktonic *E. coli* B306 (*marRAB::lacZ*) grown in CDM with carbon or magnesium limitation to high density at 37 °C with aeration.

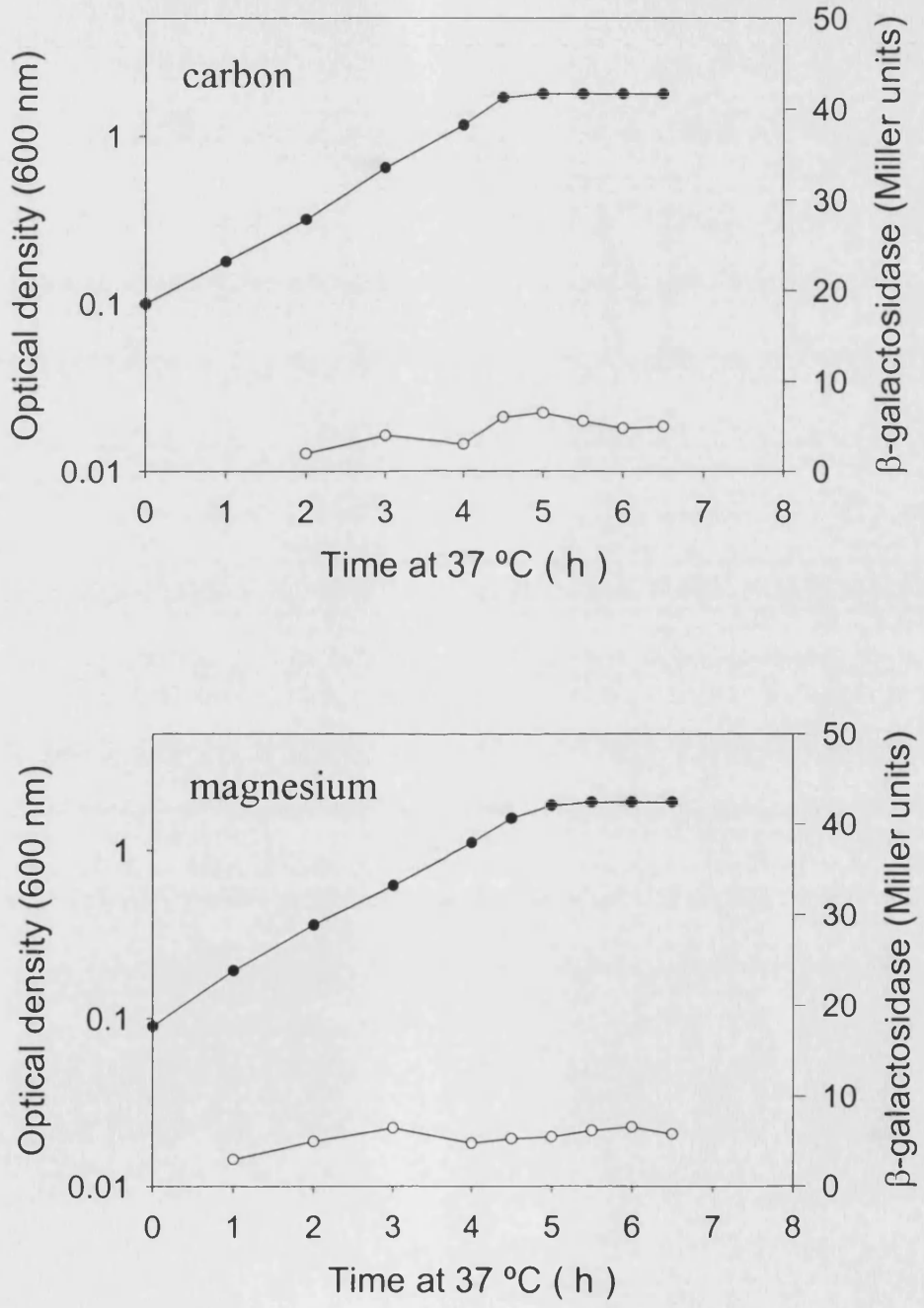
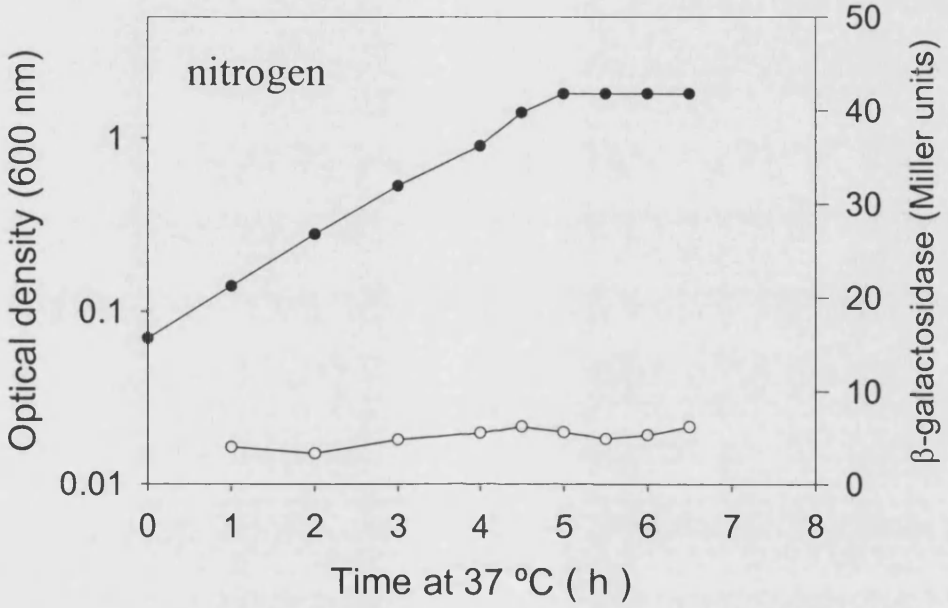


Figure 6-6. Expression of *marRAB* (open circles) throughout the growth cycle (closed circles) for planktonic *E. coli* B306 (*marRAB::lacZ*) grown in CDM with nitrogen or phosphate limitation to high density at 37 ° C with aeration.



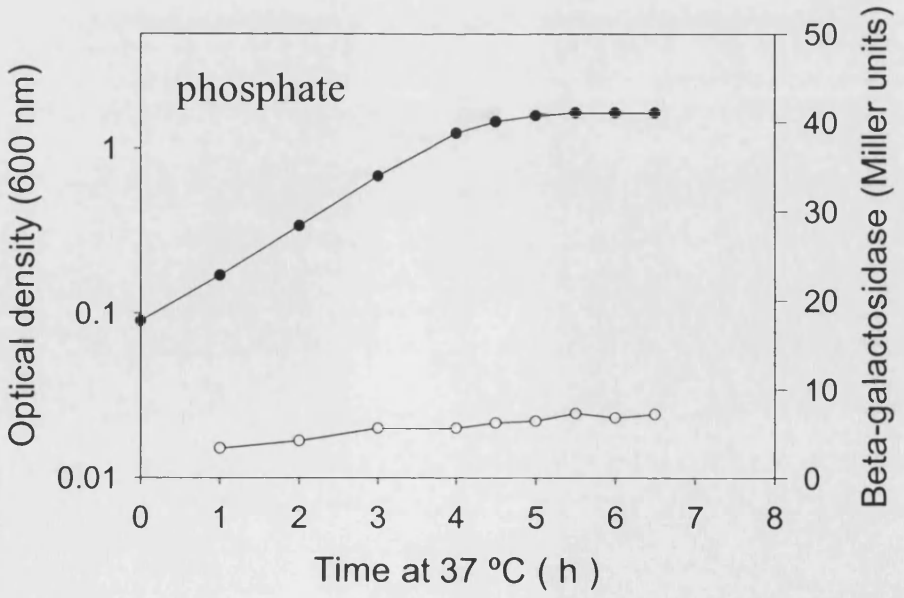


Figure 6-7. Expression of *marRAB* (open circles) throughout the growth cycle (closed circles) for planktonic *E. coli* B306 (*marRAB::lacZ*) grown in Luria-Bertani broth at 37 °C with aeration.

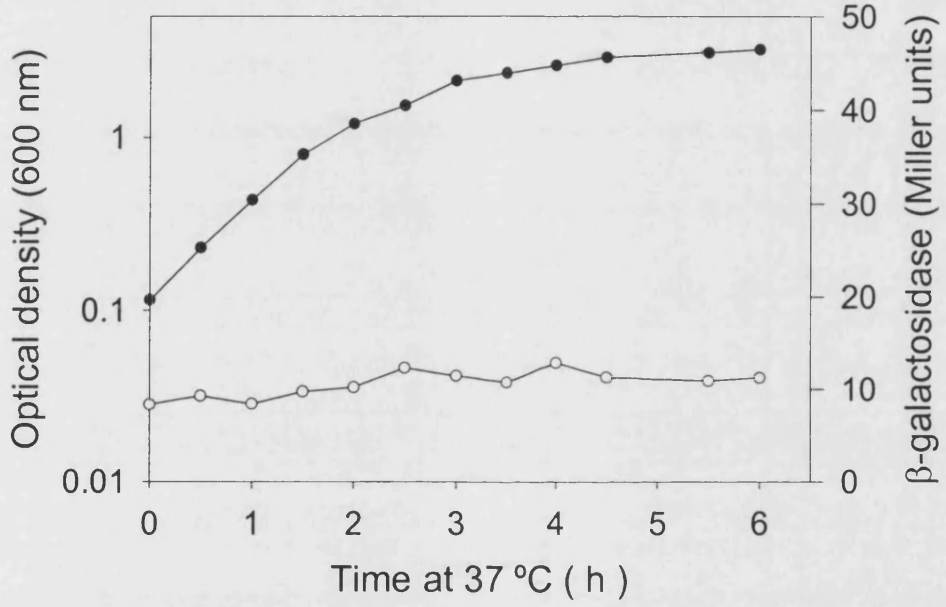
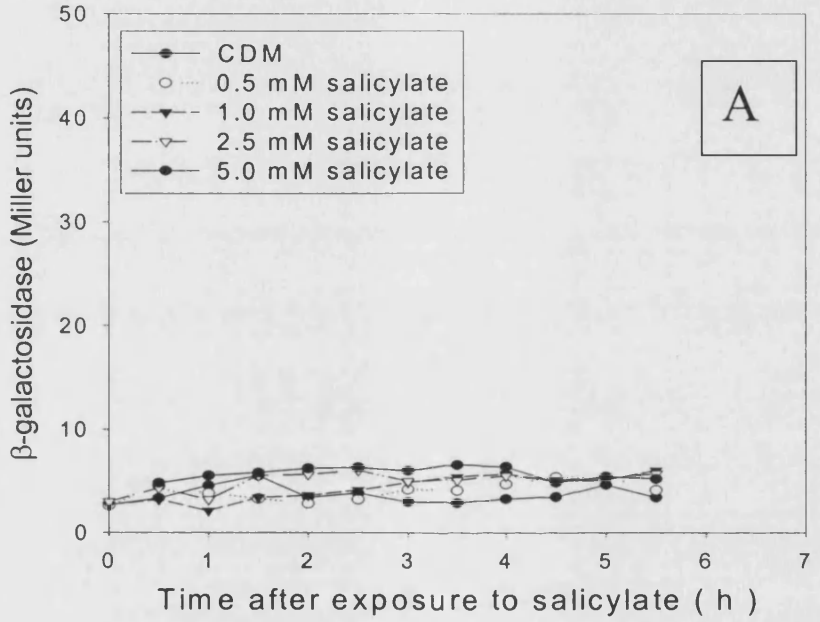


Figure 6-8. Effect of exposure to sodium salicylate during exponential phase on the expression of *marRAB* (A) and the growth of *E. coli* B306 (*marRAB*::lacZ) (B) in carbon-limited CDM at 37 °C with aeration (curves are off set for clarity).



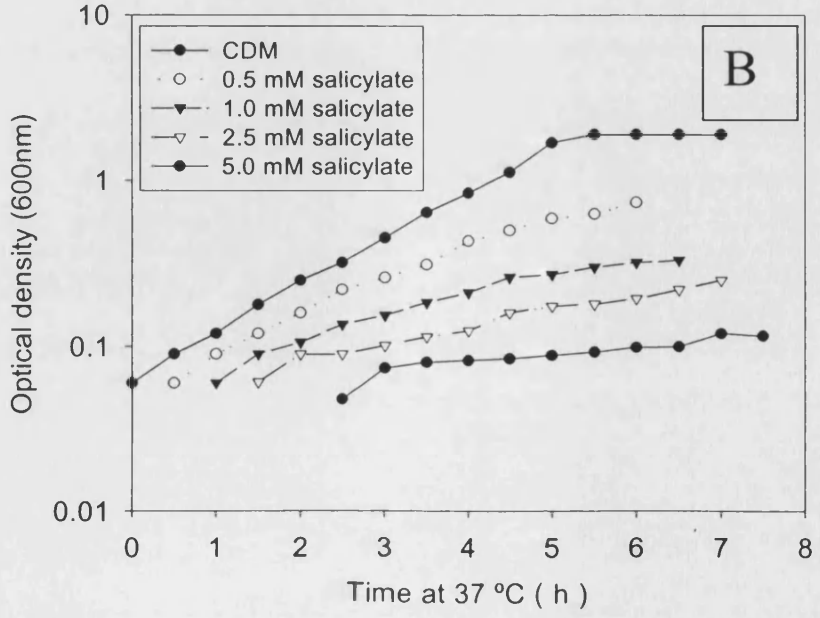


Figure 6-9. Effect of exposure to sodium salicylate during exponential phase on the expression of *marRAB* (A) and the growth of *E. coli* B306 (*marRAB*::lacZ) (B) in Luria-Bertani broth at 37 °C with aeration (curves are off set for clarity).

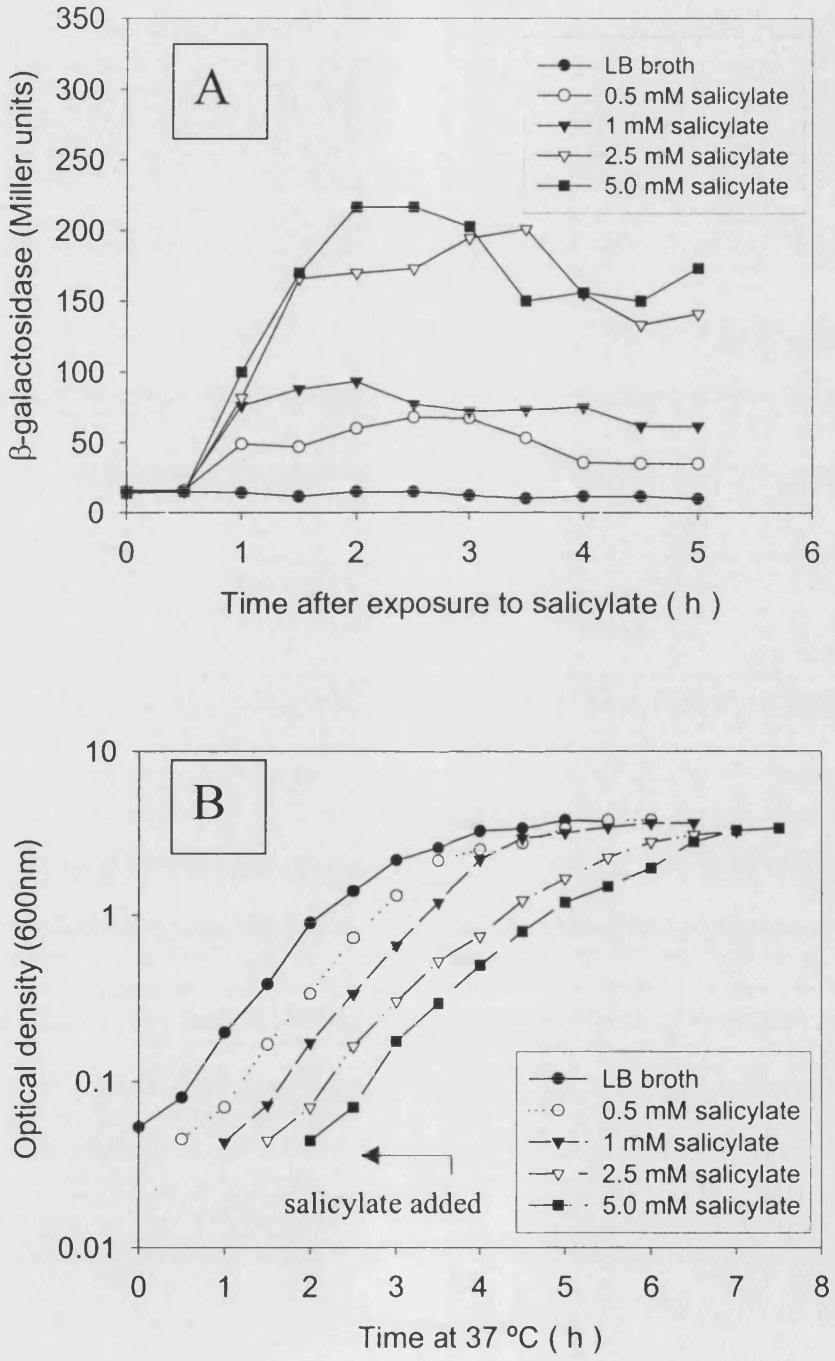


Figure 6-10. Effect of exposure to triclosan or ethanol during exponential phase on the expression of *marRAB* (A) and the growth of *E. coli* B306 (*marRAB*::lacZ) (B) in CDM with carbon limitation at 37 ° C with aeration (curves are off set for clarity).

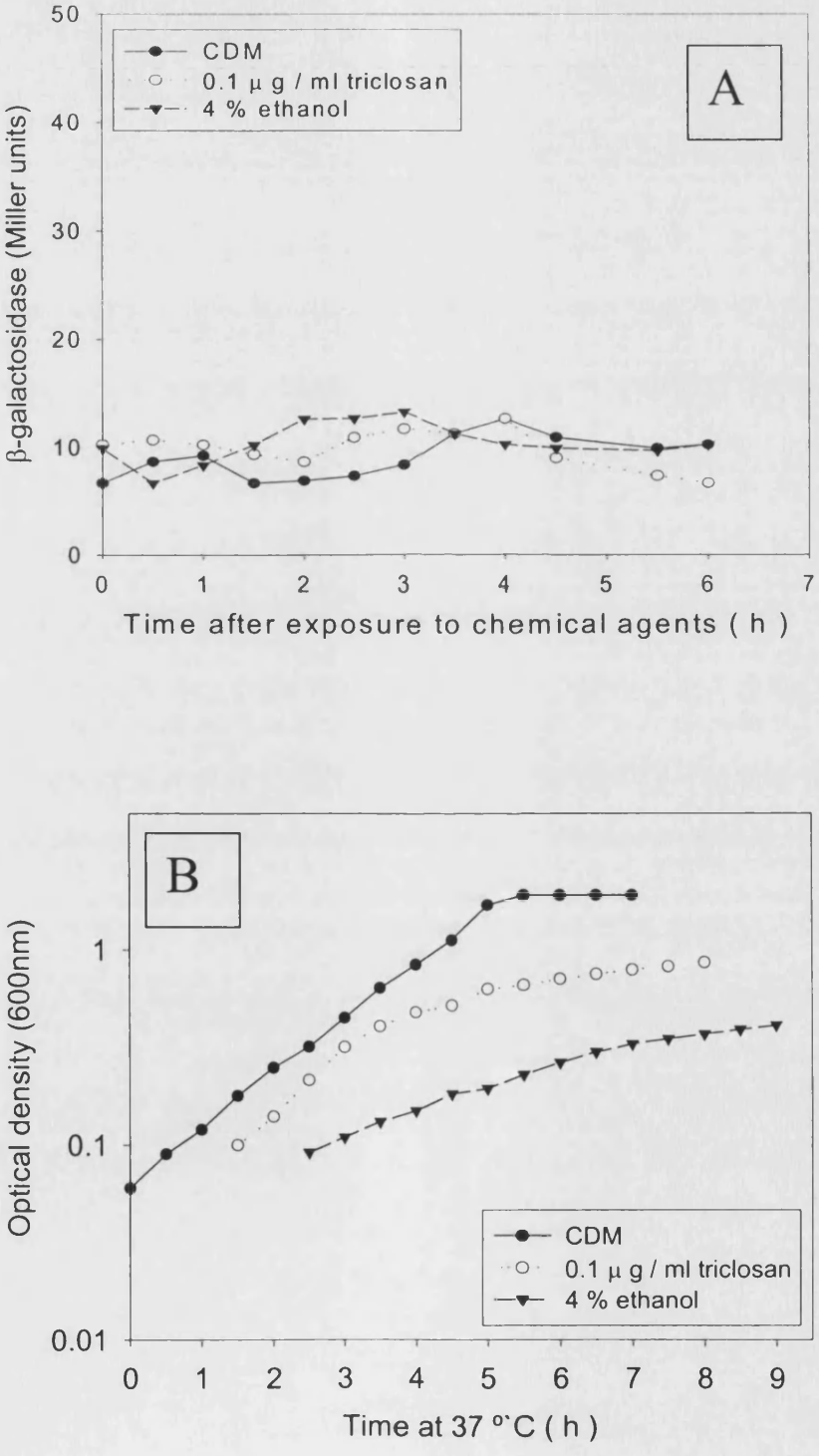
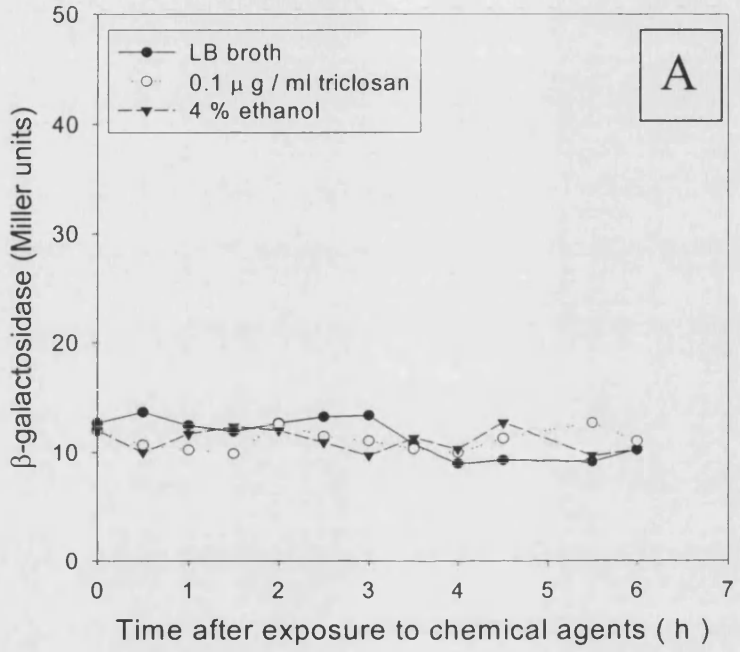
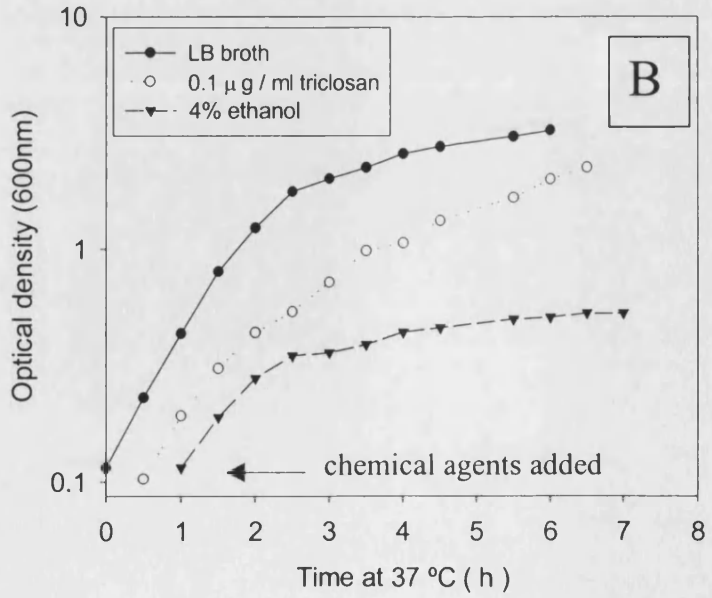


Figure 6-11. Effect of exposure to triclosan or ethanol during exponential phase on the expression of *marRAB* (A) and the growth of *E. coli* B306 (*marRAB*::lacZ) (B) grown in Luria-Bertani broth at 37 °C with aeration (curves are offset for clarity).





6.2.3 Expression of *acrAB* in response to exposure to nutrient limitation and chemical agents

The expression of the multi-drug efflux pump *acrAB* in response to nutrient limitation and exposure to various chemical agents was studied using an *acrAB* β- galactosidase reporter fusion (strain W4680) (Ma et al., 1995). When the expression of *acrAB* was studied under conditions of carbon, magnesium and nitrogen limitation it was found to be low (approximately 10 Miller units or less) (figure 6-12, 6-13). However when cells were grown in phosphate-limited CDM, expression was higher (around 20 Miller units) but there was no increase in expression concurrent with entry into stationary phase and expression was higher (compared with the other nutrient limitations) even during exponential growth (figure 6-13). The expression of *acrAB* throughout the growth cycle in LB broth was found to be comparable with that seen in carbon-, magnesium-and nitrogen-limited CDM (approximately 10 Miller units) (figure 6-14). Therefore, *acrAB* expression did not vary with growth phase in CDM or complex medium.

The expression of *acrAB* in response to exposure to triclosan (0.1 μg / ml), salicylate (5.0 mM) and ethanol (4%) was studied in both carbon-limited CDM and LB medium (figure 6-15, 6-16). The only compound found to cause *acrAB* induction was ethanol which resulted in a 4-fold and 3-fold increase in expression in CDM and LB broth respectively. Salicylate and triclosan had no effect on *acrAB* expression but did cause a decrease in growth rate in both CDM and LB.

Figure 6-12. Expression of *acrAB* (open circles) throughout the growth cycle (closed circles) for planktonic *E. coli* W4680 (*acrAB::lacZ*) grown in CDM with carbon or magnesium limitation to high density at 37 °C with aeration.

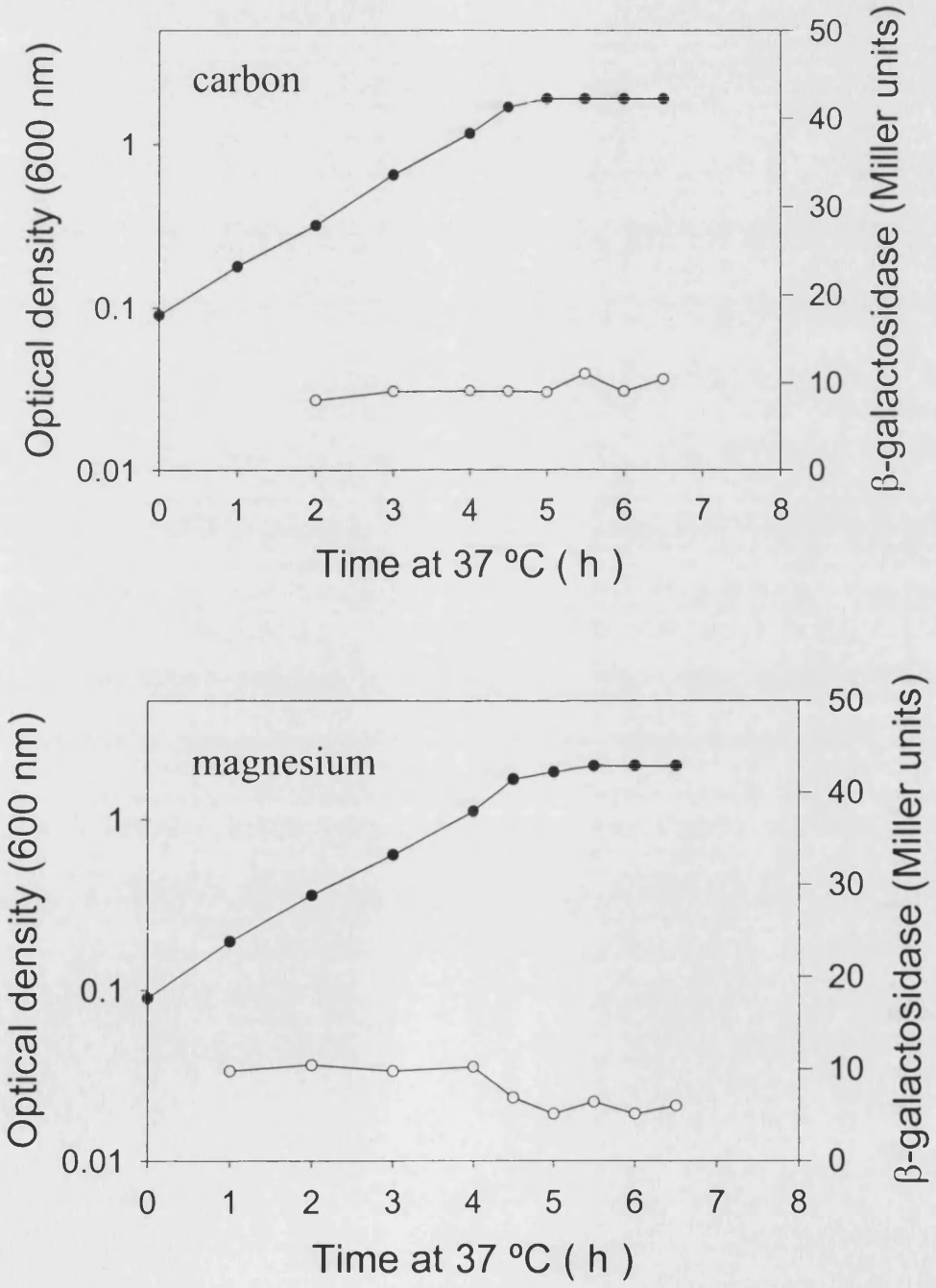


Figure 6-13. Expression of *acrAB* (open circles) throughout the growth cycle (closed circles) for planktonic *E. coli* W4680 (*acrAB::lacZ*) grown in CDM with nitrogen or phosphate limitation to high density at 37 °C with aeration.

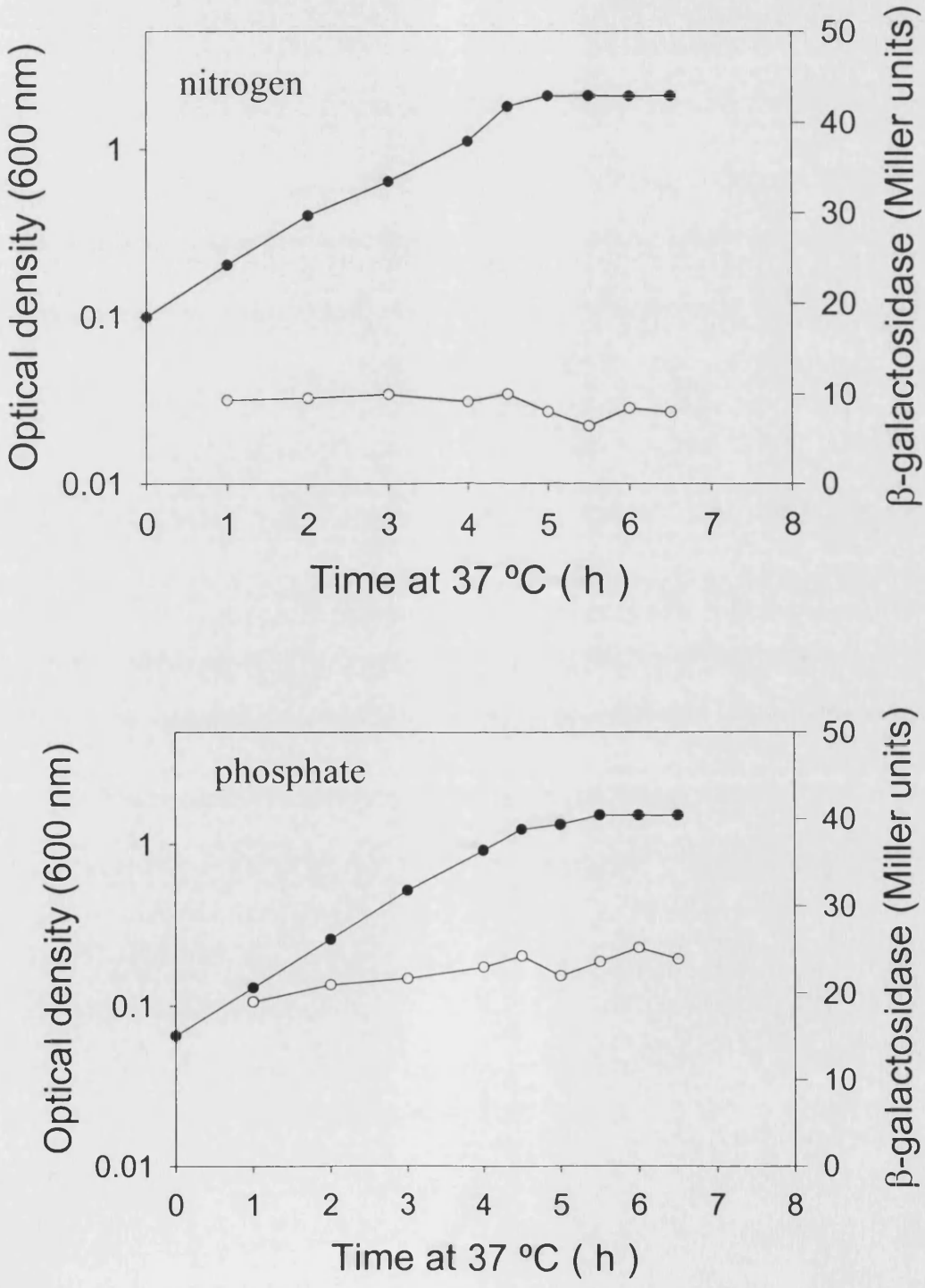


Figure 6-14. Expression of *acrAB* (open circles) throughout the growth cycle (closed circles) for planktonic *E. coli* W4680 (*acrAB::lacZ*) grown in Luria-Bertani broth at 37 °C with aeration.

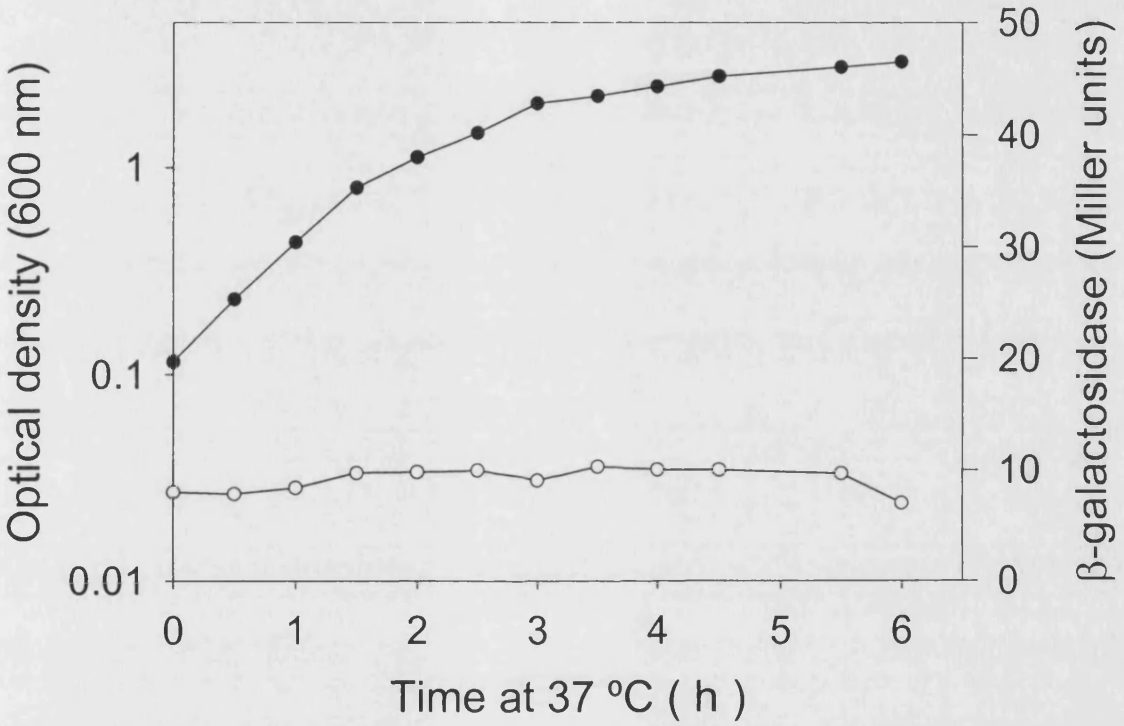


Figure 6-15. Effect of exposure to triclosan, ethanol or salicylate during exponential phase on the expression of *acrAB* (A) and the growth of *E. coli* W4680 (*acrAB*::lacZ) (B) grown in CDM with carbon limitation at 37 °C with aeration (curves are off set for clarity).

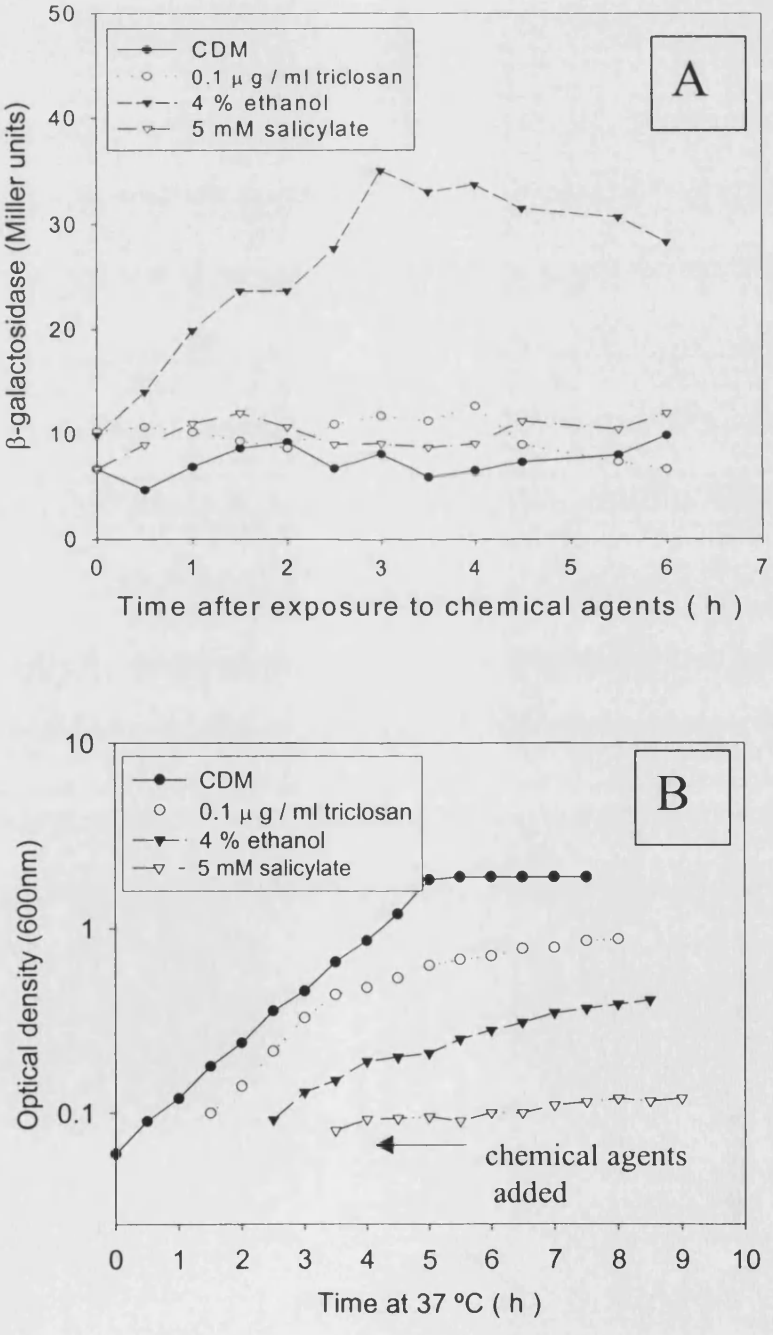
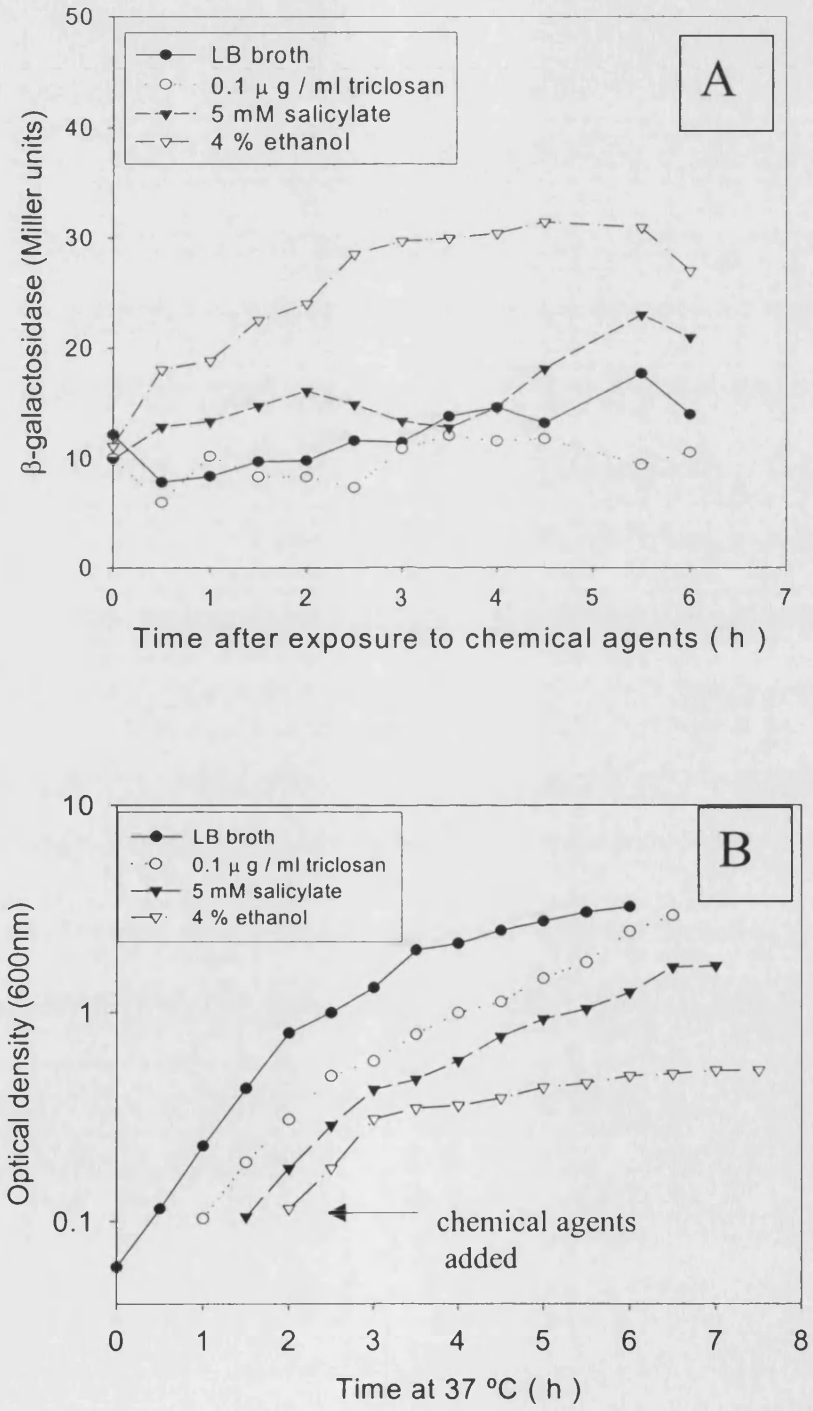


Figure 6-16. Effect of exposure to triclosan, ethanol or salicylate during exponential phase on the expression of *acrAB* (A) and the growth of *E. coli* W4680 (*acrAB*::lacZ) (B) grown in Luria Bertani broth at 37 °C with aeration (curves are offset for clarity).



6.2.4 Expression of *acrAB*, *marRAB* and *rpoS* in response to exposure to domestic products and food

The expression of *acrAB*, *marRAB* and *rpoS* in response to domestic products and food was studied using the 'ring of fire' technique developed by Gilbert (P. Gilbert, personal communication 2000). Essentially this technique involved assaying expression from a strain harbouring a β-galactosidase reporter fusion grown as a lawn on agar containing the chromogenic indicator X-gal. Domestic or food products were added to wells made in agar and gene expression determined by the production of blue colour produced by the breakdown of X-gal by β-galactosidase. Gene expression was arbitrarily scored depending on the intensity of the blue pigment produced. It was determined that there was no gene expression from the reporter fusion if no blue colouration was observed. If the blue colouration was faint (figure 6-17), gene expression was scored as low (designated by '+'). If an intense blue colour was observed (figure 6-18) gene expression was scored as high (designated by ' + + + '). Blue colouration of intermediate intensity was scored as medium (designated by '+ +').

In addition to assaying gene expression, any growth inhibition resulting from exposure to domestic or food products was measured. If no visible zone of inhibition was observed growth inhibition was scored as '-'. If the zone of inhibition was between 1 and 10 mm growth inhibition was scored as '+', between 11 and 20 mm growth inhibition was scored as '+ +' and over 20 mm growth inhibition was scored as '+ + +'.

Examination of expression of *acrAB*, *marRAB* and *rpoS* in response to a variety of domestic products produced some intriguing results (figure 6-19). Both *acrAB* and *rpoS* were induced in response to 5 out of the 8 products tested and 3 products induced *marRAB*. In response to certain products 'high-level' gene expression was observed. High level *rpoS* expression was induced in response to Colgate total toothpaste, Sainsbury's washing up liquid and Vecta bathroom cleaner. In addition, high-level *acrAB* expression resulted from exposure to Colgate Total toothpaste. However, only low-level *marRAB* expression was induced by exposure to the same product. It is interesting to note that Colgate toothpaste contains triclosan and SDS.

Zones of inhibition were seen for 7 out of the 8 domestic products tested (figure 6-19). Interestingly, gene expression only occurred if there was growth inhibition but growth inhibition was not always concurrent with gene expression. For example, Sainsbury's liquid hand soap caused a zone of inhibition of medium size but did not induce any of the reporter fusions tested.

The exposure of the reporter strains to food products also produced some interesting results (figure 6-20). The *rpoS* reporter fusion was induced by 11 out of the 13 food products tested the *acrAB* fusion by 7 products, and the *marRAB* reporter by only 2 food products. Exposure to milk induced high level expression of *acrAB*, *marRAB*, and medium level expression of *rpoS*. In addition, exposure to milk resulted in a few blue colonies scattered amongst the white (uninduced) colonies within the zone of partial inhibition (shown for the *acrAB* reporter W4680 in figure 6-18). Further studies were undertaken to determine if the isolated blue colonies were mutants exhibiting upregulation of either *acrAB* or *marRAB*. Selected potential 'mutants'

were subcultured on LB agar containing 25 % milk to ascertain if upregulation of the reporter would be maintained. After 3 consecutive subcultures colonies were transferred to LB agar overlaid with X-gal and were found to exhibit upregulation of *acrAB / marRAB*. The expression of *acrAB* and *marRAB* of the potential 'mutants' was then assayed in LB broth throughout the growth cycle (figure 6-21, 6-22). The potential 'mutants' of both *acrAB* and *marRAB* exhibited high-level (over 10-fold) constitutive upregulation of β-galactosidase even in the absence of milk compared with the wild type that had not been exposed to milk (figure 6-21, 6-22).

The effect of any mutation in the *acrAB/marRAB* reporter strains on the susceptibility to certain antimicrobial agents was determined. Initial minimum inhibitory concentration (MIC) determinations found that there was no difference between the wild type and potential 'mutants' with tetracycline, triclosan or norfloxacin. The MIC for tetracycline was 20 μg / ml for the wild type and 'mutants' of *acrAB* and *marRAB*, 0.125 μg / ml for norfloxacin and 10 μg / ml for triclosan. Subsequent studies then ascertained whether there was any difference in the kinetics of kill with these agents for wild type and 'mutant' *acrAB / marRAB* strains (results are only presented for *acrAB* wild type and 'mutant' but results obtained with *marRAB* wild type and 'mutant' were very similar). The kinetics of kill with norfloxacin (1.0 μg /ml) was identical for *acrAB* wild type and 'mutant' (figure 6-23). The *acrAB* 'mutant' was apparently slightly (approximately 0.5 log cycle) more resistant to tetracycline (15 μg / ml) (figure 6-24) and triclosan (15 μg / ml) (figure 6-25) than the wild type.

Experiments were then undertaken to determine whether alternative types of milk would also produce 'mutants' of *acrAB / marRAB*. When the *acrAB / marRAB*

reporter strains were exposed to human milk the pattern of expression was the same as for cows milk (results not shown). An intense blue ring was seen around a large zone of inhibition (20 mm), within the zone of inhibition a few (approximately 45) blue colonies were seen amongst white colonies (approximately 150). This was then repeated using Soya milk. When *acrAB / marRAB* reporter fusions were exposed to Soya milk no distinct ring of blue was seen, no zone of inhibition and no mutants (results not shown). However, there was a diffuse blue colouration across the whole of the plate of medium intensity. This was then repeated using a solution of lactose (4.7 %, the same as cows' milk-Merck index). Exposing the *acrAB / marRAB* reporter fusions to lactose produced very similar results to human and cows milk. An intense blue ring of blue was seen with a few blue 'mutant' colonies within (results not shown). Therefore, it seems feasible that a compound present in human / cow milk but absent in Soya milk is responsible for the production of *acrAB / marRAB* mutants and that this compound is likely to be lactose.

Figure 6-17. Expression of *acrAB::lacZ* reporter fusion (W4680) after exposure to (clockwise from the top) orange squash, ginger and Olivio spread as determined by the 'ring of fire' technique.

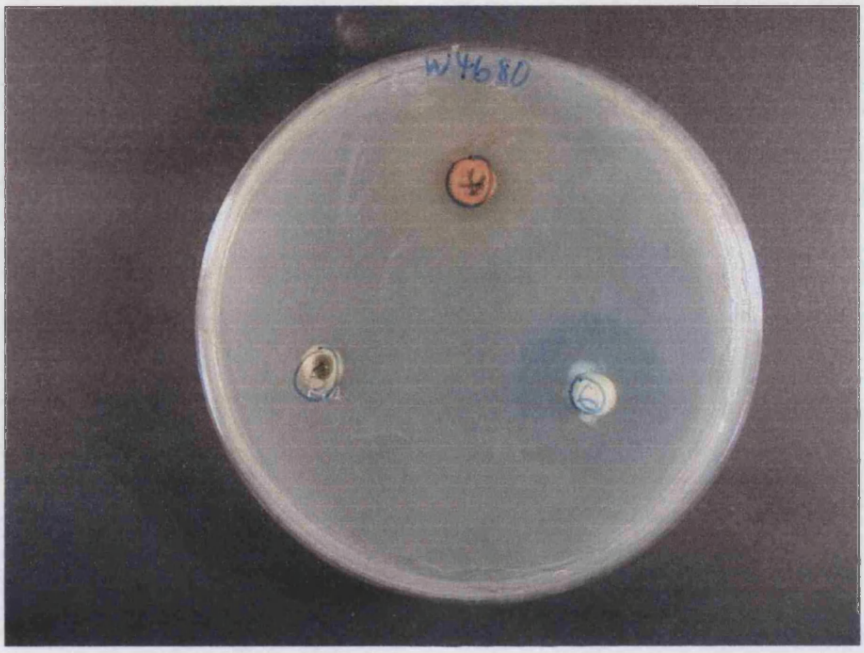


Figure 6-18. Expression of *acrAB::lacZ* reporter fusion (W4680) after exposure to (clockwise from the top) milk, coffee and mayonnaise as determined by the 'ring of fire' technique.

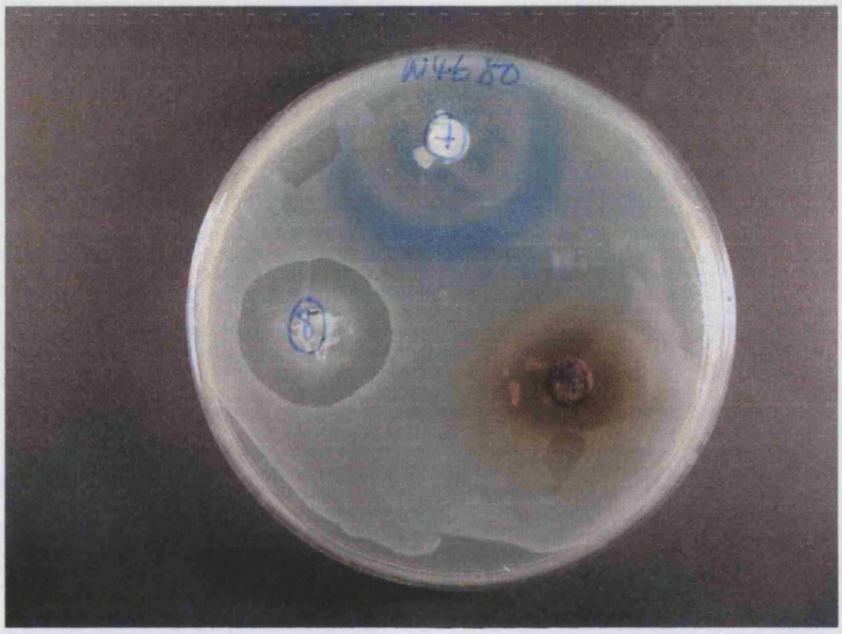


Figure 6-19. Effect of exposure to domestic products on growth inhibition and induction of *rpoS*, *acrAB* and *marRAB* for *E. coli* RO91 (*rpoS::lacZ*) W4680 (*acrAB::lacZ*) and B306 (*marRAB::lacZ*) as determined by the 'ring of fire' assay.

Product	Zone of inhibition	Induction of gene expression		
		rpoS	***acrAB***	***marRAB***
Gilette shaving foam	–	–	–	–
Colgate total toothpaste	+ + +	+ + +	+ + +	+
Safeway liquid handsoap	+ +	–	–	–
Palmolive shower cream	+ + +	+	+ +	–
Imperial leather moisturising handwash	+ + +	–	+	+ +
Kindwash hand-washing detergent	+ +	+	+	–
Sainsbury's washing up liquid	+ +	+ + +	–	–
Vecta bathroom cleaner	+ +	+ + +	+ +	+ +

Figure 6-20. Effect of exposure to food products on growth inhibition and induction of *rpoS*, *acrAB* and *marRAB* for *E. coli* RO91 (*rpoS::lacZ*). W4680 (*acrAB::lacZ*) and B306 (*marRAB::lacZ*) as determined by the 'ring of fire' assay.

Product	Zone of inhibition	Induction of gene expression		
		rpoS	***acrAB***	***marRAB***
Fresh ginger	–	+	–	–
Chili flakes	–	+	+	–
Heinz tomato ketchup	+ +	+	+ +	–
Lime pickle	–	++	+ +	–
Olive oil	–	+	–	–
Olivio spread	–	+	+	–
Milk (semi-skimmed)	–	+ +	+ + +	+ + +
Garlic	+ + +	+ + +	–	+
Coffee	–	–	–	–
Orange squash	–	–	–	–
Mayonnaise	+ +	+ +	+ +	–
Tea	–	+	–	–
Red wine	+	+	+ + +	–

Figure 6-21. Induction of *acrAB*::lacZ reporter fusion (triangles) throughout the growth cycle (circles) in *E. coli* W4680 wild type (*acrAB*::lacZ) (closed symbols) and potential mutant (obtained after exposure to milk) (open symbols) grown in Luria-Betani broth at 37 °C with aeration.

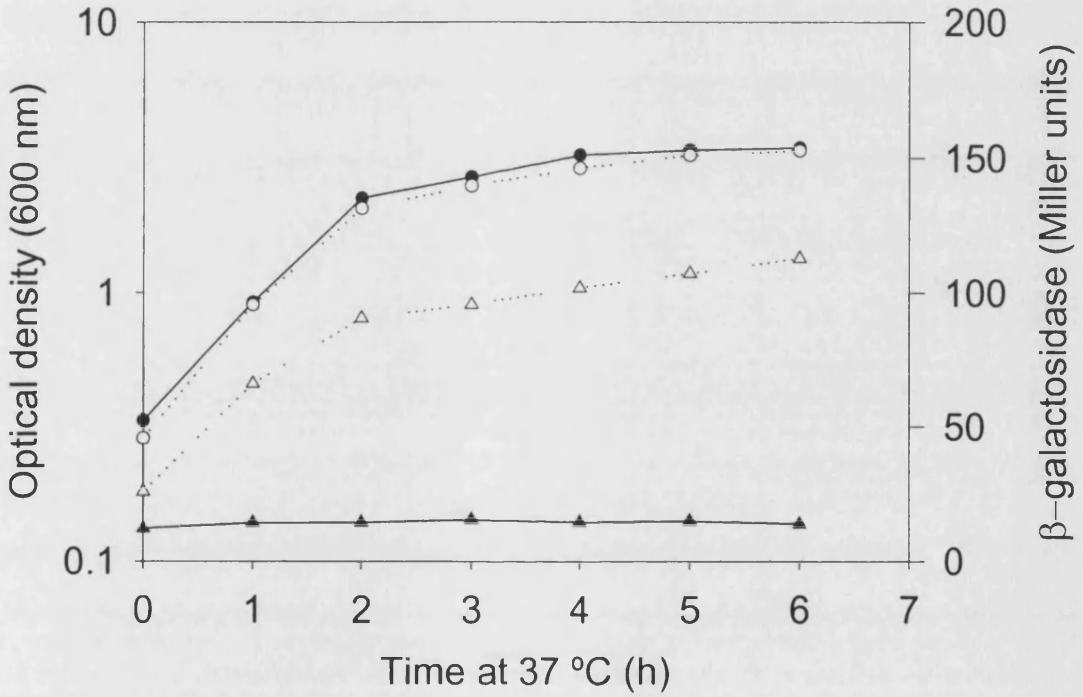


Figure 6-22. Induction of *marRAB*::lacZ reporter fusion (triangles) throughout the growth cycle (circles) in *E. coli* B306 wild type (*marRAB*::lacZ) (closed symbols) and potential mutant (obtained after exposure to milk) (open symbols) grown in Luria-Betani broth at 37 °C with aeration.

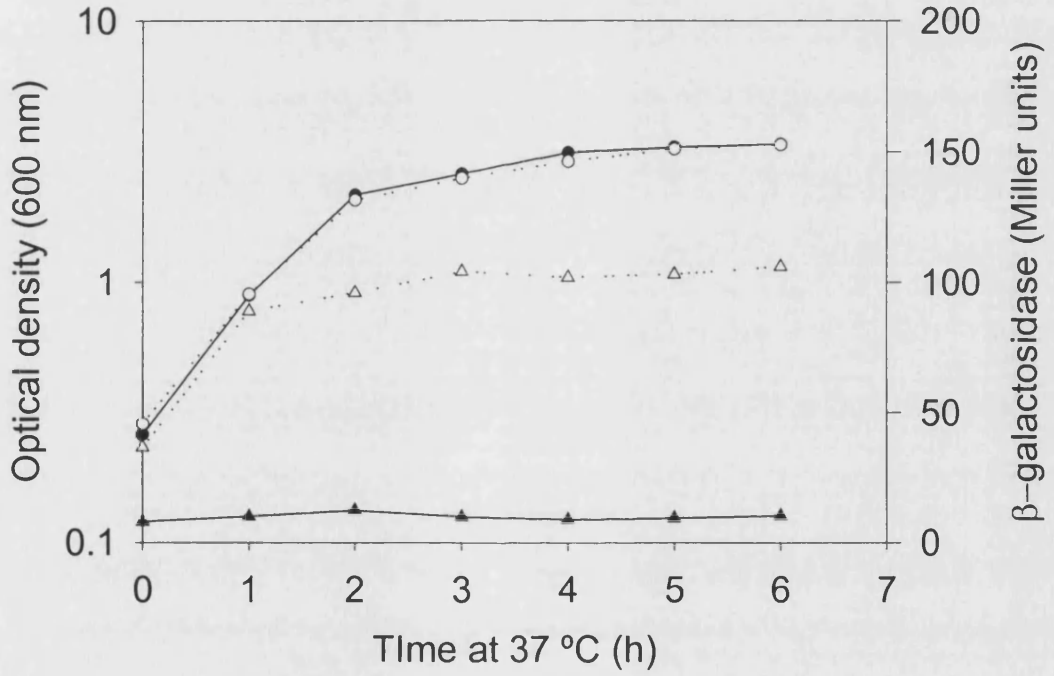


Figure 6-23. Effect of exposure to norfloxacin (1.0 μg / ml) on the induction of *acrAB::lacZ* reporter fusion in exponential phase (4 generations before stationary phase) *E. coli* W4680 wild type (*acrAB::lacZ*) (closed circles) and potential mutant (obtained after exposure to milk) (open circles) grown in Luria-Bertani broth at 37 ° C with aeration.

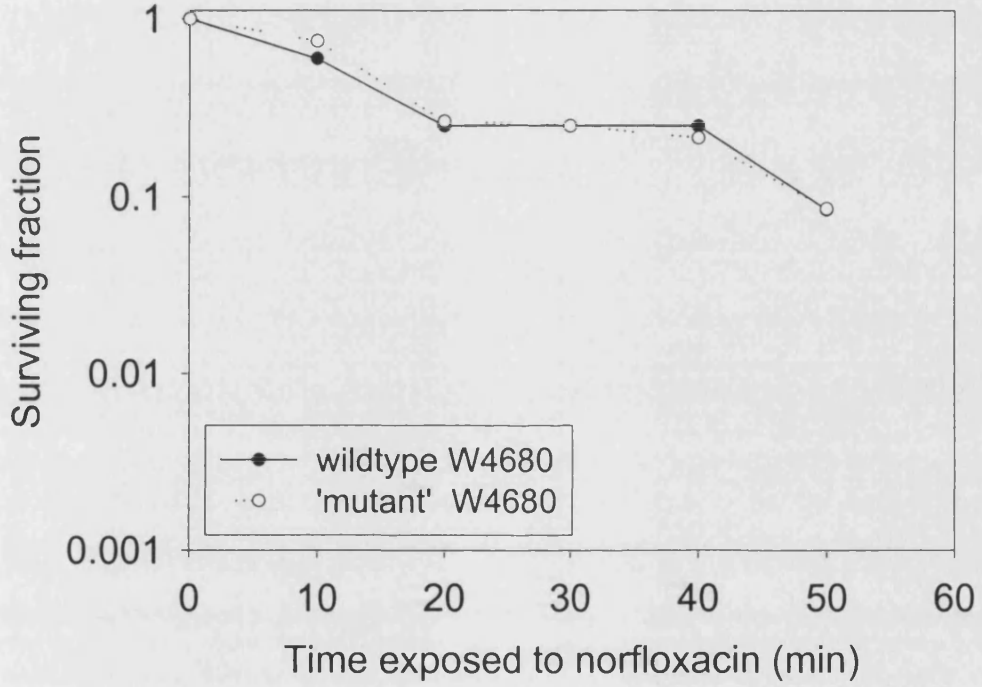


Figure 6-24. Effect of exposure to tetracycline (15 μg / ml) on the induction of *acrAB::lacZ* reporter fusion in exponential phase (4 generations before stationary phase) *E. coli* W4680 wild type (*acrAB::lacZ*) (closed circles) and potential mutant (obtained after exposure to milk) (open circles) grown in Luria-Bertani broth at 37 °C with aeration.

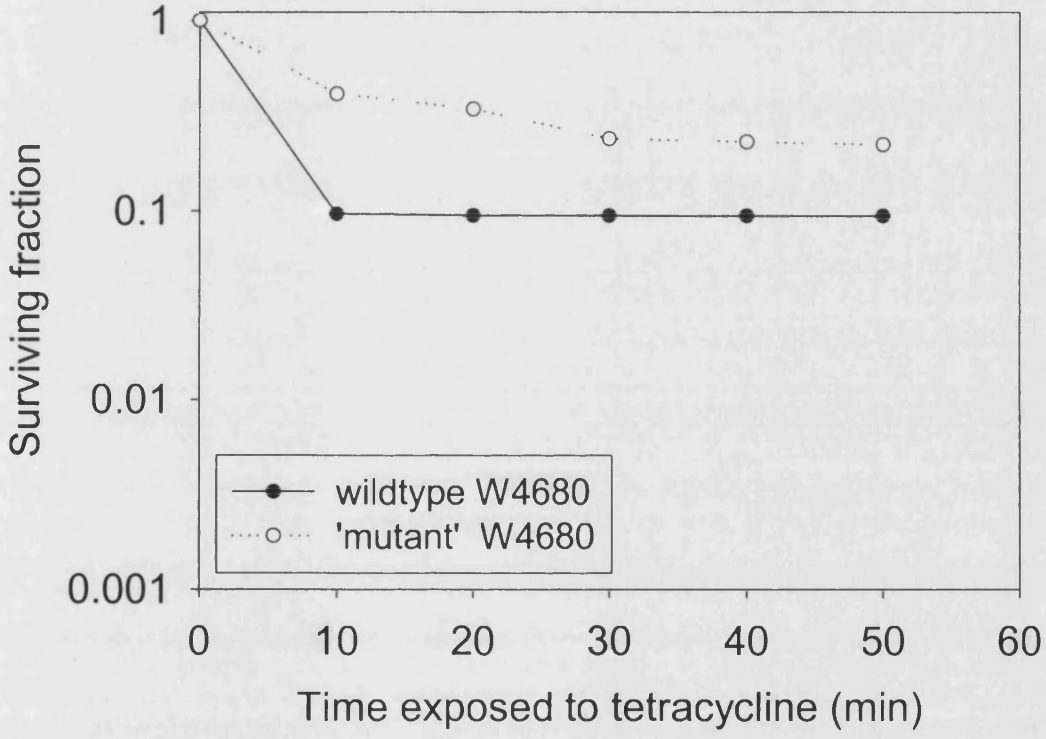
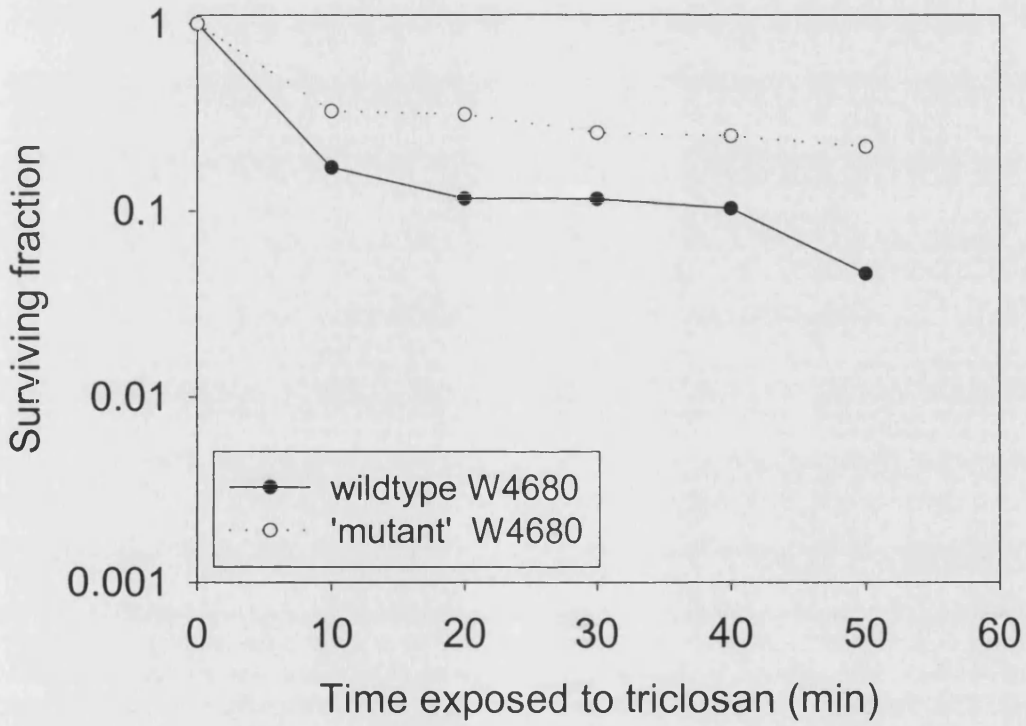


Figure 6-25. Effect of exposure to triclosan (15 μg / ml) on the induction of *acrAB::lacZ* reporter fusion in exponential phase (4 generations before stationary phase) *E. coli* W4680 wild type (*acrAB::lacZ*) (closed circles) and potential mutant (obtained after exposure to milk) (open circles) grown in Luria-Bertani broth at 37 ° C with aeration.



6.3 DISCUSSION

6.3.1 Expression of *rpoS* in response to chemical agents

RpoS was found to be induced by all of the chemical agents tested in this study. Moderate expression of *rpoS* occurred after exposure to norfloxacin, tetracycline and benzalkonium chloride. The high level induction of *rpoS* in response to triclosan was particularly interesting. The effect of triclosan on the induction of other stress responses has been a topic of much recent study (McMurry et al., 1998). However, its influence on *rpoS* has not previously been determined. There appeared to be some correlation between the change in growth rate caused by exposure to antimicrobial agents and *rpoS* induction. This was particularly apparent in response to triclosan and tetracycline where *rpoS* was induced only if the concentration of the agent was sufficiently high to cause a reduction in growth rate. It is feasible that the link between reduction in growth rate and *rpoS* expression may act via the positive effector of *rpoS*, the alarmone ppGpp (Gentry et al., 1993). ppGpp provides a direct connection between *rpoS* and growth rate in the starved *E. coli* cell (Teich et al., 1999; Chatterji and Kumar Ojha, 2001) and it is possible that ppGpp may influence *rpoS* expression in response to exposure to other stresses, including antimicrobial agents. However, large perturbations in growth rate did not always lead to a concomitant increase in *rpoS* expression. In response to exposure to the highest concentrations of tetracycline or norfloxacin tested, *rpoS* expression was not induced or only induced to a low level. It is possible that under these circumstances the antimicrobial agents were at such a high concentration that the cells were suffering irreparable damage and were unable to initiate the induction of *rpoS*. As tetracycline is an inhibitor of protein synthesis, it is not surprising that *rpoS* was not induced in response to high concentrations of this agent.

RpoS regulated events may lead to cells that are not only more resistant to various stresses but also more virulent (Hengge-Aronis, 2000). Therefore, it is possible that the induction of *rpoS* in response to antimicrobial agents could be cause for concern. However, it is unlikely that induction of *rpoS* caused by exposure to antimicrobial agents is significant in terms of public health since bacteria in the domestic environment are likely to be subjected to numerous additional stresses that would also cause *rpoS* induction.

6.3.2 Expression of *acrAB* and *marRAB* in response to chemical agents

AcrAB and *marRAB* were overall, difficult to induce in batch culture. In response to all of the chemical agents tested, *marRAB* was induced only by salicylate. Previous studies have also shown salicylate to be a potent inducer of the *marRAB* operon (Cohen et al., 1993). Interestingly *marRAB* induction occurred only if the cells were grown in complex medium. Exposure of cells to salicylate in carbon-limited CDM did not lead to *marRAB* induction. Exposing CDM-grown cells to salicylate resulted in a much more severe perturbation of growth rate compared with LB grown cells. It is feasible that exposure to salicylate was more damaging to CDM-grown cells and prevented *marRAB* induction. However, this seems unlikely, as the lowest concentration of salicylate tested did not result in complete cessation of growth although it did significantly reduce the growth rate. It would be interesting to determine whether exposure of CDM-grown cells to lower concentrations of salicylate would cause *marRAB* induction. Salicylate is thought to enable the activation of the *marRAB* operon by binding the repressor protein MarR preventing it from binding to the operator region (*marO*) thus enabling transcription of the activator protein MarA (Martin and Rosner, 1995). It is thought that MarA enables the

activation of antibiotic resistance-associated genes such as *acrAB* and *micF* as it binds a region of their promoters, the 'mar box', thus enabling upregulation of these genes (Barbosa and Levy, 2000). However, as *acrAB* is not induced in response to salicylate there must be an additional level of regulation that determines when *marRAB* regulated genes are induced.

Expression of the *acrAB* efflux system was induced only in response to 4 % ethanol. As *marRAB* was not induced by ethanol this suggests that that induction of *acrAB* may act independently of *marRAB*. This finding is in agreement with previous studies that have also found that the induction of *acrAB* in response to ethanol occurs independently of *marRAB* / soxRS (Ma et al., 1995; Ma et al., 1996). Interestingly, the repressor of the *acrAB* operon, *acrR* is upregulated in tandem with *acrAB* in response to ethanol (Ma et al., 1996). This indicates that a complex network (acting independently of *marRAB*) must counteract *acrR* repressor activity and enable the activation of *acrAB*. The mechanism of this regulation is yet to be elucidated.

Interestingly, triclosan did not cause induction of either *acrAB* or *marRAB*. This finding was surprising, as triclosan is known to be a substrate for *acrAB* and over-expression of *acrAB* or *marRAB* leads to resistance to triclosan in clinical and laboratory strains of *E. coli* (McMurry et al., 1998). However, it is important to note that the effect of triclosan on *acrAB* / *marRAB* expression has not been studied previously. It is feasible that the presence of the true pump inducer (bile salts) (Thanassi et al., 1997) may be required in order to induce high-level *acrAB* expression. A similar mechanism of induction is reported for the *bmr* and *blt* efflux

pumps of *Bacillus subtilis* which have highly similar substrate specificities but require different natural inducers (Ahmed et al., 1995).

The expression of *marRAB* and *acrAB* throughout the growth cycle was also studied. Neither *acrAB* nor *marRAB* showed any increase in expression throughout the growth cycle in LB or nutrient-limited CDM. Interestingly, it has previously been reported (using the same *acrAB* reporter fusion as in this study) that *acrAB* exhibits growth phase regulated expression and is induced upon entry into stationary phase in rich medium (Ma et al., 1995). However, close analysis indicates the small increase in *acrAB* expression reported (2.5 fold) occurred four generations the onset of stationary phase and therefore may not be linked to entry into stationary phase (Ma et al., 1995). However, it is important to note that stationary phase-associated events may begin to occur several generations before the onset of stationary phase (Buhler et al., 1998). It is also interesting to note that a subsequent study by the same authors stated that there was no increase in *acrAB* expression if cells were grown in carbon-limited minimal medium and that expression did not increase in LB supplemented with glucose (Ma et al., 1996). This study provided the novel finding that *acrAB* expression is higher when cells are grown in CDM with a limiting concentration of phosphate. This increased expression did not seem to be directly linked to phosphate limitation as expression was higher throughout the growth cycle when cells were grown in CDM with a limiting concentration of phosphate.

The present study found no significant change in *marRAB* expression throughout the growth cycle in either CDM or LB medium. However, a previous study has indicated that in CDM, *marRAB* expression is maximal in log phase and decreases upon entry

into stationary phase and conversely, that expression is maximal in stationary phase and minimal in mid-log in rich media (Maira-Litran et al., 2000b). However, it is important to note that the reporter fusion used in the previously reported study (Maira-Litran et al., 2000b) differs from that used in this study. The precise nature and construction of a reporter fusion may dramatically alter the perceived gene expression and makes comparisons between different reporter fusions difficult. Also, it is difficult to make reliable comparisons without an understanding of the specific transcriptional start site and whether the introduced reporter fusion has interfered with post-transcriptional regulatory mechanisms (Pessi et al., 2001). The effect of alternative reporter fusions on the calculated level of gene expression is apparent if the basal level of gene expression is considered. The *marRAB* reporter fusion (B306) utilized in this study had a basal level of expression of approximately 10 Miller units compared with the reporter used in a previous study which had a basal level of 255 to 560 miller units (Maira-Litran et al., 2000b). In addition, it is noteworthy that although the previous study stated that there was variation in *marRAB* expression throughout the growth cycle the actual level of change was less than 2-fold (Maira-Litran et al., 2000b).

6.3.3 Induction of *rpoS*, *acrAB* and *marRAB* by domestic products and food as determined by the 'ring of fire' technique

The 'ring of fire' technique enabled simple, rapid, visual analysis of gene expression from lacZ reporter fusions in response to exposure to food and domestic products. However, the technique was limited in its application as it does not enable quantitative analysis of gene expression. Nevertheless, it did provide some particularly interesting data. A number of food and domestic products were very

potent inducers of the stress systems studied. In particular, Colgate toothpaste caused very strong induction of both *rpoS* and *acrAB* and low-level induction of *marRAB*. It is interesting to note a component of Colgate toothpaste, triclosan, has already been shown to cause high-level *rpoS* induction in batch culture in this study. However, batch culture exposure to triclosan did not lead to *acrAB* expression in this study. It is feasible that higher concentrations of triclosan or differing test conditions may have influenced the expression of *acrAB*. Alternatively, another component of Colgate toothpaste may be responsible for the induction of *acrAB* seen in the 'ring of fire' assay. SDS is an ingredient of many domestic products, including Colgate toothpaste and it is a substrate of the *acrAB* efflux pump (Ma et al., 1995). It is possible that SDS could contribute to the high-level *acrAB* induction seen after exposure to Colgate toothpaste.

The extent of the antimicrobial activity of the domestic products used in this study was readily apparent using the 'ring of fire' technique. A moderate to large zone of inhibition resulted from exposure to 7 of the 8 domestic products tested in this study. Interestingly, inhibition of growth and induction of stress genes was not limited to exposure to domestic products. Many food products used in this study exhibited strongly inhibited microbial growth and were potent inducers of *acrAB*, *marRAB* or *rpoS*. The antimicrobial activity of garlic, tomato ketchup, red wine and mayonnaise was particularly notable. Garlic has traditional dietary and medicinal applications as an antimicrobial agent. A recent study indicated that garlic oil sulphides had substantial antibacterial activity against many pathogenic enteric bacteria including *E. coli* (Ross et al., 2001). Red wine may exhibit antimicrobial activity because of its ethanol content and as a result of bacteriocin production by lactic acid bacteria present

(Navarro et al., 2000). It is feasible that the high acetic acid content of tomato ketchup could lead to growth inhibition.

Interestingly, the vast majority of food products resulted in expression of at least one of the stress genes studied. This is slightly surprising as *acrAB* and *marRAB* proved to be largely uninducible by chemical agents in planktonic culture and yet were highly induced by exposure to food products such as tomato ketchup, lime pickle, red wine and milk. The response of the *acrAB* and *marRAB* reporter fusions to exposure to milk was particularly noteworthy. It well documented that milk may possess antimicrobial properties as the high concentration of lactoferrin effectively removes available iron making microbial growth difficult (iron restriction reviewed in Hantke and Braun, 2000). This study (Chapter 3) has already indicated that growth in medium containing low levels of iron may lead to high-level *rpoS* induction. However, the effect of low iron on *acrAB* and *marRAB* has not been studied although *marRAB* is linked to the regulation of several iron uptake and transport genes (Barbosa and Levy, 2000). Although it is feasible that exposure to milk may constitute a 'stress' and result in the induction of *acrAB*, *marRAB* and *rpoS*, the selection of potential 'mutants' after exposure to milk was surprising. However, as the 'mutants' did not possess a true multiple antibiotic resistant phenotype (they were not substantially more resistant to antimicrobial agents known to be substrates of the *acrAB* efflux pump) it is likely that any mutation was not in either *acrAB* or *marRAB*. Additionally, as 'mutants' could also be produced after exposure to lactose it is feasible that any mutation is in the β- galactosidase component of the *acrAB* / *marRAB* reporter fusion.

The strain background for the *acrAB/marRAB* reporter fusions lacks a functional β-galactosidase operon (Ma et al., 1995; Gambino et al., 1993). Therefore, β-galactosidase is only produced concurrent with induction of *acrAB/marRAB*. It is possible that any mutation is within the *lacZ* component of the reporter fusion or that a reversion has occurred in the background strain *lac* operon. These mutations may enable constitutive expression of β- galactosidase so that lactose can be utilized as a carbon source. Interestingly, the phenomenon of selection for mutations in the *lac* operon following exposure to lactose has been a subject of much recent study (Foster, 2000; Hendrickson et al., 2002). It has been demonstrated that when certain strains lacking a functional *lac* operon reach stationary phase in the presence of lactose, non-growing cells appear to direct mutations preferentially to sites that enable the utilization of lactose. This phenomenon is referred to as 'adaptive mutation' (Foster, 2000; Hendrickson et al., 2002). It would be useful to sequence both the *acrAB / marRAB::lacZ* reporter and the background *lac* operon to determine if the potential 'mutants' isolated in this study had mutations in the *lac* operon.

In summary, this study indicates that exposure to biocides in the domestic environment is unlikely to lead to high-level induction of either *acrAB* or *marRAB*. Bacteria in the domestic environment are likely to exist in a non / slow-growing, nutrient-limited state as a biofilm. This study has shown that conditions of nutrient limitation did not lead to upregulation of *acrAB/marRAB* and previous studies have shown that biofilm growth does not cause induction of *acrAB* or *marRAB* (Maira-Litran et al., 2000a; Maira-Litran et al., 2000b). Additionally, this study has shown that both *acrAB* and *marRAB* are more strongly induced by exposure to common food products than many domestic products or biocides.

It is important to note that this study has only examined the phenotypic induction of stress genes in response to biocides and not the selection of mutants with constitutive upregulation of *acrAB* or *marRAB*. Recent studies have indicated that exposure to certain biocides in both the laboratory and clinical setting may lead to the production of mutants in *acrAB / marRAB* that exhibit multiple antibiotic resistance (Oethinger et al., 2000; McMurry et al., 1998; Nikaido, 1998). However, in the absence of biocides, overexpression of efflux systems can be deterimental as it leads to the efflux of important cellular metabolites and slows growth (Nikaido, 1998). It seems likely that in the absence of selective pressure, any mutation in *acrAB* or *marRAB* would be lost.

It also seems likely that mutations other than those in *acrAB* or *marRAB* should be cause for greater concern. The biocide triclosan is unusual in that it has a specific cellular target, the fatty acid biosynthesis enzyme enoyl [acyl carrier protein] reductase (*fabI*) (McMurry et al., 1998b). Strains exhibiting mutations in the *fabI* target gene have been isolated and they exhibit 100-fold greater resistance to triclosan than strains with mutations in *acrAB/ marRAB* (McMurry et al., 1998b). Mutations in *fabI* also confer cross-resistance to other antimicrobial agents (Heath et al., 1999) for example, in *M. tuberculosis* it may also result in resistance to isoniazid (Slayden et al., 2000; Parikh et al., 2000), a drug widely used to treat tuberculosis.

7 CONCLUDING REMARKS

This study has examined the relative contribution of *rpoS*-regulated events to the response of *E. coli* to stress. Overall, *rpoS* was not essential for the survival or adaptation of *E. coli* to stress conditions. Generally, the specific nutrient limitation was a much stronger influence on the susceptibility of cells to stress and the physiological adaptations to stress than *rpoS*. Despite recent studies that have implied that *rpoS* is almost exclusively responsible for cell survival under non-optimal conditions (reviewed in Hengge-Aronis, 2000), this study found that cells lacking *rpoS* did not die as soon as they were exposed to stress but, under many circumstances showed similar survival to cells expressing *rpoS*. It is not surprising that *rpoS*-regulated GSR is not essential for survival under stress conditions as many additional stress responses exist. There are specific stress responses for heat shock (Arsene et al., 2000; Yuru et al., 2000)), DNA damage (Walker et al., 2000), nitrogen-starvation (Reitzer and Schneider, 2001), acid shock (Foster, 2000) and cold shock (Phadtare et al., 2000), all of which are autonomous from *rpoS*. It is likely that there are complex interconnections between many of the stress responses and that this gives an additional level of refinement to the response of *E. coli* to stress. In addition, it could almost be considered an 'evolutionary dead end' or at the least a very risky strategy if cells relied solely on *rpoS* for stress survival as any mutation that rendered *rpoS* inactive would leave the cells highly susceptible to all stress. Likewise, it is possible that *rpoS* has evolved as a mechanism to coordinate stress responses but as it has no true promoter specificity (Lonetto et al., 1992; Tanaka et al., 1995), in its absence other sigma factors can transcribe *rpoS*-regulated genes.

In addition, any conditions where expression of *rpoS* conferred some advantage to planktonic cells (such as response of cells to heat or the production of trehalose) could be negated by growth as a biofilm. Therefore, it seems unlikely that the GSR plays a significant role in the recalcitrance of biofilms. Biofilm growth appeared to override any requirement for *rpoS* and it was certainly not essential for resistance to any of the stress conditions tested. Therefore, this study has provided some support for the theory that biofilms exhibit a distinct phenotype with different regulation of stress-associated genes compared with planktonic cells. This study has also indicated that it is likely that there is not just a single biofilm phenotype and that multiple biofilm phenotypes exist depending on specific environmental conditions including (but not solely) nutrient limitation and cell density. It is feasible that additional global regulators or other sigma factors are induced under conditions of biofilm growth and that they enable differential regulation of resistance genes. Identification of biofilm-specific genes could play an important role in the development of novel antimicrobial agents to combat biofilms.

This study also examined the induction of *marRAB* and *acrAB* under stress conditions. The findings of this study provided no support for the theory that exposure to biocides could lead to induction of *marRAB* / *acrAB* and the expression of a multiple antibiotic resistance phenotype in the domestic environment (Russell, 1999; Schweizer, 2001). When cells were exposed to stresses likely to be encountered in the domestic environment *acrAB* and *marRAB* were largely uninducible. In fact, both genes were much more strongly induced by exposure to common foodstuffs such as milk. In addition, bacteria in the domestic environment are likely to be existing as an adherent biofilm. It is known that the contribution of

acrAB / *marRAB* to biofilm resistance is negligible (Maira-Litran et al., 2000a; Maira-Litran et al., 2000b) and it is likely that the induction of a specific biofilm phenotype is much more significant in terms of recalcitrance and resistance to antimicrobial agents.

7.1 SUGGESTIONS FOR FUTURE WORK

1. Comparison of total proteins produced under conditions of nutrient-limited planktonic and biofilm growth using 2D-PAGE. This would be invaluable in determining important components of a biofilm phenotype and any biofilm specific regulation. A similar approach could involve utilizing microarray technology and examining transcript differences between planktonic and biofilm growth.

2. Determination of inorganic polyphosphate levels in nutrient-limited biofilm and planktonic cells.

3. Clarification of the role of *rpoS* in LuxS-mediated quorum sensing.

4. Investigation of the role of the nitrogen-starvation associated sigma factor (σ^{54}) in general resistance mechanisms including resistance to antimicrobial agents.

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